The effect of winter grazing and a nitrification inhibitor on nitrous oxide emissions and denitrification in a stony soil

---

A thesis

submitted in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy

at

Lincoln University

by

Glen Treweek

Lincoln University

2015

Abstract of a thesis submitted in partial fulfilment of the

requirements for the Degree of Doctor of Philosophy.

The effect of winter grazing and a nitrification inhibitor on nitrous oxide emissions and denitrification in a stony soil

by

Glen Treweek

Intensive grazing of forage crops by dairy cows is common practice during the winter months in the Canterbury region of New Zealand. Such intensive grazing on wet soil increases the risk of denitrification and the production of the powerful greenhouse gas, nitrous oxide (N_2O). However, at the beginning of this research project, no information was available on denitrification and N_2O emission from forage crops grown in free-draining stony soils, which are widespread in Canterbury. The objectives of this thesis were, therefore, to quantify N_2O emissions from winter grazed forage soil, develop methods to mitigate those emissions, and to improve knowledge and understanding of the effects of severe animal trampling on the physical properties of, and denitrifying microbial population in, the soil below forage crops grazed by dairy cows during winter.

Three field-based experiments were carried out in 2011, 2012 and 2013, and were complimented by a laboratory-based experiment in 2012. The field experiments used lysimeters and soil blocks collected from a stony Balmoral soil used for forage kale. The nitrification inhibitor, dicyandiamide (DCD), and a carbon-rich biochar were also used as soil amendments to mitigate N_2O emissions.

Total N_2O emissions from trampled soil ranged from 1.3% to 1.9% of urine-N applied to the soil. When applied at 20 kg ha^{-1}, DCD reduced total N_2O emissions from trampled soil with urine by 44-68%, but DCD did not significantly affect N_2O emissions from non-trampled soil. Biochar, with or

without DCD, had no significant effect on N_2O emissions when incorporated into the soil at 5 t ha^{-1} (1% of soil dry weight).

Trampling increased the bulk density of the fine earth fraction of the stony soil from 0.89 g cm^3 to 1.11 g cm^3 ($P<0.05$) in year two, and from 1.03 g cm^3 to 1.19 g cm^3 ($P<0.05$) in year three. Trampling decreased the air permeability of the stony soil by more than one order of magnitude ($P<0.01$). When sampled, the volumetric water content of the trampled soil was, on average, higher than that of the non-trampled soil ($P<0.05$), which combined with the reduction in air permeability to create anaerobic conditions suitable for the growth of denitrifying microbes.

Significant increases in the abundance of NO_2^- reducing *nirS* gene copies were observed in trampled soil that had been treated with urine (under both laboratory and field conditions). Similarly, in the field, significantly more N_2O reducing *nosZ* clade-I ($P<0.05$) and *nosZ* clade-II ($P<0.01$) gene copies were found in trampled soil than non-trampled soil. The increase in *nosZ* gene copies in urine amended trampled soil was accompanied by an increase in the proportion of ^{15}N labelled urine emitted as N_2 gas – the final product of denitrification ($P<0.05$). The increase in denitrifying gene copies and corresponding increase in urine-derived N_2 gas following trampling is an important finding, as at the time of writing, no authors had described such a relationship.

This work has shown that denitrification does occur in free draining stony soil when forage crops are intensively grazed by dairy cows during winter, and is an important N-loss pathway that should not be overlooked.

Keywords: animal, dairy, winter, forage, grazing, kale, fodder, urine, trampling, treading, pugging, poaching, compaction, N^{15}, bulk density, air permeability, denitrifiers, *nirS*, *nirK*, *nosZ*

Contents

Tables

Figures

Chapter One – Introduction

The advent of refrigerated shipping in the 1880's saw the beginning of large scale dairy exports from New Zealand (NZ) (Philpott 1937). Dairy farming is economically important for NZ. For example, in the year ended June 2014, dairy products accounted for more than 25% of NZ's export earnings (Statistics New Zealand 2014). However, the contribution of dairy farming to the NZ economy has come at an environmental cost. Diffuse losses of nitrogen (N) from grazing dairy cattle have been identified as having adverse environmental effects, both on the quality of water bodies, and the composition of the atmosphere (Ministry for the Environment 2013, Wright 2013).

In the Canterbury region of NZ, the dairy sector has seen rapid expansion in both the size and number of dairy farms in the past two decades (Ministry for the Environment 2013). Dairy farming in NZ is traditionally based on a low-cost, low-input, pasture-based system, where cows are grazed outdoors year round (Figure 1.1) (de Klein and Ledgard 2001, Drewry *et al.* 2008, Ministry for the Environment 2011, Luo *et al.* 2013b). To maximise milk production of the modern dairy herd in NZ, animal calving, and subsequent peak milk production and feed requirements, are timed to coincide with maximum pasture growth in the spring (Kleinman *et al.* 2008). However, during winter in the cooler southern regions, the non-lactating dairy herd is commonly grazed off-farm, to preserve the pasture for the spring when feed demand is highest (de Klein and Ledgard 2001, Drewry and Paton 2005, Smith *et al.* 2008a).

Figure 1.1: Young dairy cows grazing pasture in New Zealand.

Crop and animal management practices typically differ between on-farm pasture-based dairy farming, and off-farm winter forage grazing. High yielding forage crops, such as brassica, are often utilised in off-farm winter grazing regimes, and such systems when used on dairy farms are here termed the winter dairy forage system (WDFS). To efficiently utilise the high yielding crops, animal stocking rates on the WDFS are much higher than they otherwise would be on pasture, and are normally grazed by cows in large mobs or fed in narrow strips across the paddock (Figure 1.2) (Monaghan *et al.* 2007, Thomas *et al.* 2008). In contrast to stock densities of approximately 70-90 cows ha^{-1} when grazing pasture (Moir *et al.* 2011), stock densities when grazing on the WDFS are frequently in the range of 300 - 800 cows ha^{-1} (Drewry and Paton 2005, Pleasants *et al.* 2007, Drewry *et al.* 2008).

Trampling damage to the soil under such high stock densities is more likely during wet winter conditions, with some soils more prone to trampling damage than others. Soils with a high stone content are common on the alluvially-deposited Canterbury plains (Carrick *et al.* 2013), and these stony soils provide an ideal base for winter forage grazing, as the stony soil matrix provides greater resistance to trampling damage than fine textured soil (Batey 2009). About 1.7 million hectares of stony soils have been mapped in NZ, and more than half of those are located in the Canterbury region (Carrick *et al.* 2013). Rapid drainage and infiltration, together with low water holding capacity and low nutrient retention capacity typify these stony soils

Figure 1.2: Dairy cows strip-grazing forage kale at Lincoln University's Ashley Dene farm during winter.

Ashley Dene, where this research was conducted, is a 350 ha partially irrigated farm owned by Lincoln University. Ashley Dene was a dryland sheep farm for about 100 years prior to the introduction of winter forage grazing in 2010 (Figure 1.3). Most of the soils at Ashley Dene have been mapped as

the stony Balmoral series, classified as Pallic Orthic Brown Soil (Hewitt 2010); Udic Haplustept loamy skeletal (Soil Survey Staff 2010), which contains up to 60% stones in the top 20 cm, overlying sandy gravels (Webb and Bennett 1986). Despite the stony nature, observations have revealed that the Balmoral soil can suffer substantial damage when the winter forage crops are grazed by dairy cows (Figure 1.4), which may favour denitrification, and high rates of nitrous oxide (N_2O) emission (Luo *et al.* 2010).

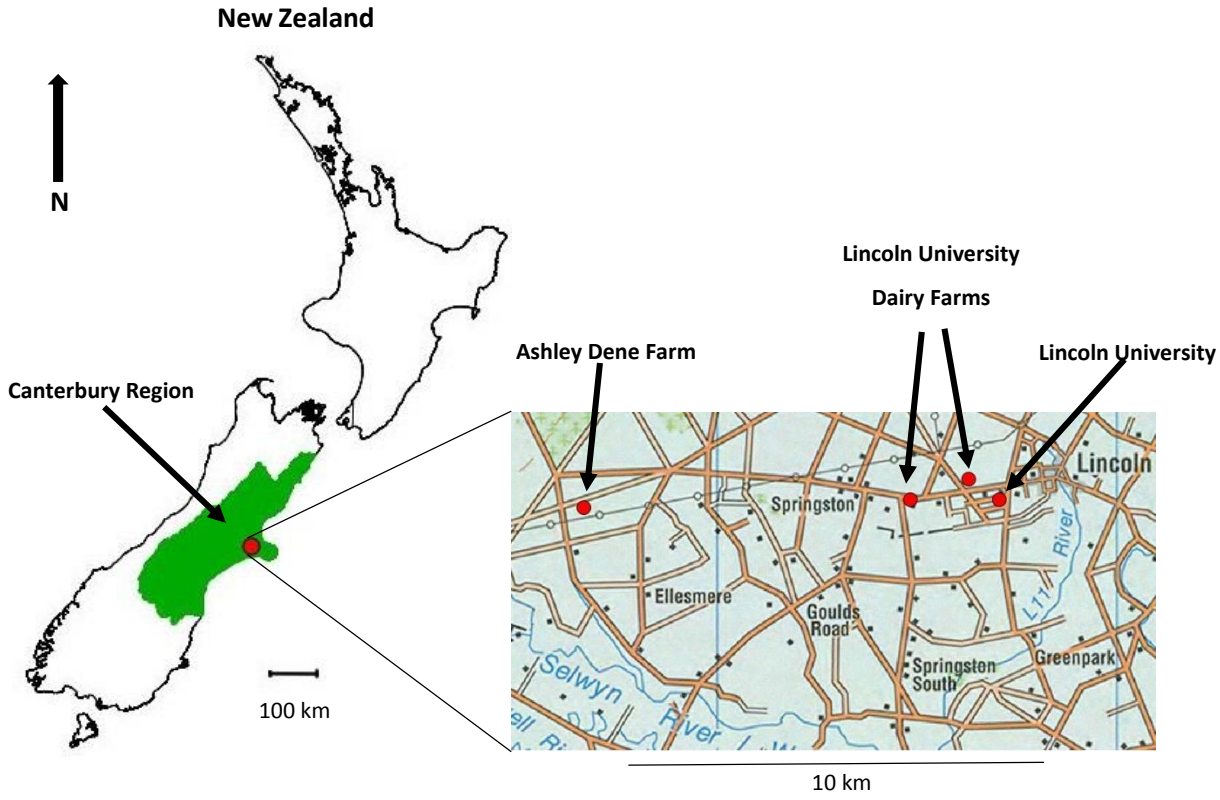

Figure 1.3: **Study setting. The Canterbury region (in green) is situated in the South Island of New Zealand. Ashley Dene Farm (43°38'55"S, 172°20'39"E), where this study is focused, lies approximately 10 km west of Lincoln University (43°39'04"S, 172°28'03"E) (inset).**

Nitrous oxide is a powerful greenhouse gas that has also been linked with stratospheric ozone destruction. Studies have indicated that the amount of N_2O emitted from winter grazing on slow draining finer textured soil can be considerable (e.g. Luo *et al.* 2008c, Smith *et al.* 2008b, Thomas *et al.* 2008, Monaghan *et al.* 2013). However, extensive animal trampling damage is not normally associated with free draining stony soil, and no published research exists to describe N_2O emissions from winter forage grazing on stony soil, or ways to mitigate those emissions. Soil amendments such as nitrification inhibitors and organic carbon 'biochar' have been used successfully to mitigate N_2O emissions from other agricultural systems (e.g. Di and Cameron 2002, 2008, Luo *et al.* 2010, Zhang *et al.* 2010, Taghizadeh-Toosi *et al.* 2011), but there is little information on their efficacy in a WDFS.

Figure 1.4: **Damage to the stony Balmoral soil following grazing of a forage crop by dairy cows at Lincoln University's Ashley Dene Farm.**

The key hypothesis defining the research of this thesis was: The potentially high substrate availability (i.e. urine), and animal trampling effects in a winter dairy forage system, will favour denitrifier activity and high rates of N_2O production via denitrification, which can be reduced by the application of DCD and biochar.

Chapter Two of this thesis reviews previously published research relevant to N_2O emissions from denitrification following winter forage grazing. Chapter Three describes the experimental design components of this thesis. Chapters Four through Seven describe the research experiments, and in Chapter Eight, the results are discussed and future research suggested.

Chapter Two – Literature Review

2.1 Introduction

Greenhouse gas (GHG) emissions from New Zealand (NZ) have increased by about 25% between 1990 and 2012, with the majority of those emissions coming from the agricultural sector. Nitrous oxide (N_2O) has been identified as a key agricultural GHG, and urine deposition by grazing animals recognised as a major source of N_2O. About 97% of NZ's N_2O is produced by the agricultural sector, and the NZ government has pledged to make reductions in net national GHG emissions (Ministry for the Environment 2014).

Winter dairy support blocks typically utilise high yielding forage crops, where animal stocking densities are much higher than they otherwise would be on pasture. High animal stocking rates concentrate animal urine deposition, and increase the likelihood of soil damage from animal trampling, which may increase the amount of N_2O emitted. Although dairy cows are often taken to graze winter forage crops in farming systems in NZ, this review of the literature shows that there has been little research into the amount of N_2O emitted through denitrification from a winter forage grazing system, or ways to mitigate those emissions.

2.2 Greenhouse gas overview

Greenhouse gases are the components of the Earth's atmosphere that permit life on Earth by retaining some of the energy emitted from the sun as heat (Steinfeld *et al.* 2006, IPCC 2007, de Klein *et al.* 2008). Observations have shown an increase in global surface temperatures during the 20th Century (IPCC 2013). Numerous authors have concluded that anthropogenically produced GHG's are responsible for the warming trend, which may lead to a range of effects, such as sea level rise and mass species extinctions, while some fear the future of humanity may be threatened by catastrophic and irreparable changes to global weather patterns (e.g. Le Treut *et al.* 2007, de Klein *et al.* 2008, IPCC 2013).

In 1998 New Zealand (NZ) adopted the Kyoto protocol (de Klein *et al.* 2001). The intention of the Kyoto protocol was to create international measures that reduced the risk of potential global climate change, through reducing anthropogenic GHG emissions (de Klein et al. 2008). Like many other Kyoto signatories, the NZ Government set a target of reducing GHG emissions to 1990 levels (Ministry for the Environment 2011). Despite the Kyoto protocol, GHG emissions from NZ have continued to increase, by an estimated 25% between 1990 and 2012 (Ministry for the Environment 2014). Nitrous

oxide (N_2O) is an important greenhouse gas, as it has a long atmospheric residence time when compared with other GHG's (Prather and Hsu 2010). Nitrous oxide emissions have increased globally since the beginning of the industrial revolution (Figure 2.1), and in NZ, N_2O emissions have increased by about 32% between 1990 and 2011 (Davidson 2009, Ministry for the Environment 2014).

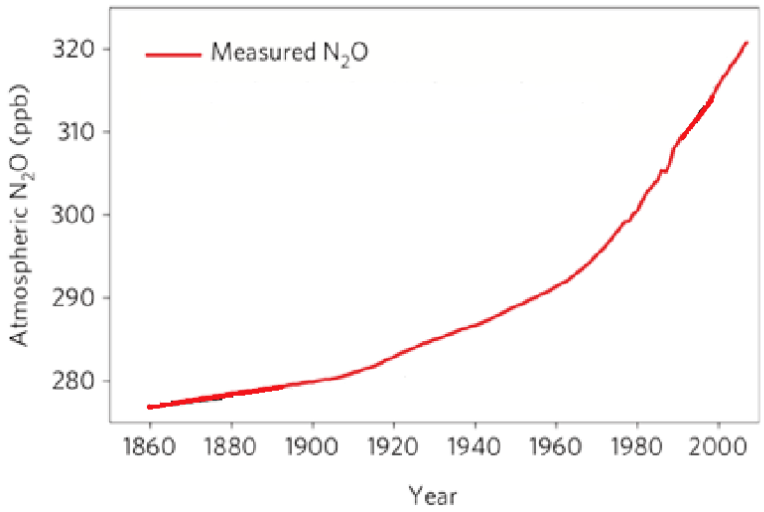

Figure 2.1: Increase in atmospheric N_2O since 1860 (adapted from Davidson 2009).

Although the NZ Government has not committed to the second phase of the Kyoto process, in agreement with many other Kyoto signatories, NZ has adopted an emissions trading scheme (ETS), where those whose activities produce net positive GHG emissions will be required to purchase emission allocation units (Ministry for the Environment 2011). While most global GHG emissions come from energy production and industry, in 2012, the largest producer of GHG emissions in NZ was the agricultural sector (Ministry for the Environment 2014). In 2012, N_2O comprised 14.3% of all GHGs from NZ – twice the global average – with about 97% of that N_2O coming from agricultural soil (IPCC 2013, Ministry for the Environment 2014). The agricultural sector in NZ is not yet required to trade GHG units in the ETS, however, the sector is required to report biological GHG emissions produced on farms. Accurate data are therefore essential to ensure that GHG emissions are correctly reported, and to identify potential areas where economical reductions in GHG emissions can be made.

2.3 Nitrogen in soil

Nitrogen is required by all living organisms to build amino acids, synthesise proteins, and transfer energy. However, about 98% of Earth's N is locked up in rocks and minerals, or bound to primary silicates in the Earth's crust. Of the 2% of N remaining on Earth, more than 95% is found in the atmosphere as the relatively inert N_2 gas. A very small proportion of the balance of global N is found in the soil, which usually comprises between 0.1% and 0.6% N (Cameron *et al.* 2013). Most soil N is in the organic form and not readily available to plants (Figure 2.2). Consequently, N is often the limiting nutrient if sufficient water is available to support plant growth.

Much of the naturally occurring soil N has accumulated following the symbiotic relationship between select microorganisms and leguminous plants in a process termed fixation (Freiberg *et al.* 1997, Jetten 2008). In addition to biological fixation, the advent of the Haber-Bosch process has allowed substantial amounts of synthetically fixed N to be added to agro-ecosystems (Galloway 1998, Mosier *et al.* 1998, Galloway *et al.* 2003) (Figure 2.2).

Naturally occurring or synthetically produced organic N can be mineralized in soil, which is a microbially driven process where proteins and other organic compounds are broken down to produce ammonia (NH_3) (Haynes 1986, Galloway *et al.* 2003). Ammonia is readily hydrolysed to ammonium (NH_4^+) which is a form available to plants and other organisms. Ammonium is oxidised by nitrifiers for energy, and in doing so, nitrate (NO_3^-) is produced. Once N is mineralized to an inorganic form, it becomes mobile within terrestrial, aquatic, and atmospheric ecosystems (Galloway *et al.* 2003). Ammonium and NO_3^--N can rapidly be synthesised to organic compounds in soil and become part of the organic N pool, a process termed immobilisation (Williams and Haynes 1994, Booth *et al.* 2005).

Several authors have comprehensively described the N cycle in pastoral agriculture (e.g. Haynes and Williams 1993, Whitehead 1995, Ledgard *et al.* 2009, Cameron *et al.* 2013).

When ingesting sufficient plant matter to meet energy needs, the N content of most plants exceeds the requirements of grazing animals. Consequently, it is common for 60-90% of the N ingested by dairy cows to be excreted in dung and urine (Haynes and Williams 1993). When animals consume high-N feed, most of the excess N is excreted in the urine, rather than the faeces (Lantinga *et al.* 1987). Dairy cows have been estimated to urinate about 8-12 times per day (Haynes and Williams 1993, Aland *et al.* 2002, Dennis *et al.* 2011), which can provide a substantial amount of N to the soil in small, localised spots, which are here termed urine patches (Figure 2.3). The concentration of N under a dairy cow urine patch can be the equivalent of up to 1,000 kg N ha^{-1} (Cameron *et al.* 2013).

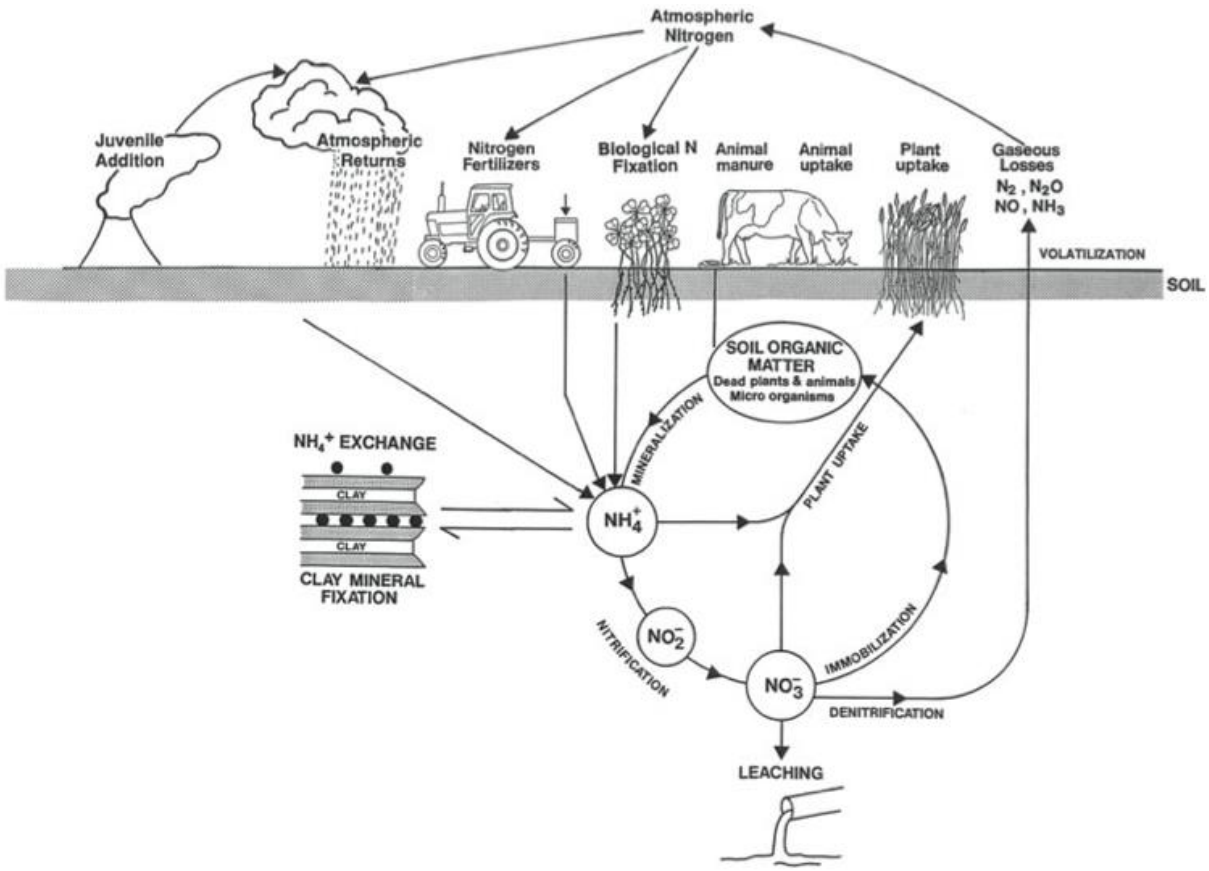

Figure 2.3: The soil/plant nitrogen cycle (Cameron 1992).

The fate of N excreted by dairy cows is dependent on a number of factors. The majority of N contained in dung is bound in organic compounds and becomes slowly available, whereas the N in urine is mainly in the form of urea ($CO(NH_2)_2$), which is rapidly hydrolysed to NH_4^+, and then nitrified to NO_3^-(Haynes and Williams 1993, Oenema *et al.* 1997, Cameron *et al.* 2013). Therefore, urine-N is more mobile than faecal-N. For example, plants have been found to absorb more urine-N than faecal-N (Saarijarvi and Virkajarvi 2009), and for the same given N-rate, more N_2O was emitted from soil when urine was applied than when faeces was applied (Luo *et al.* 2013a). With the majority of excreted-N found in a cow's urine, and the higher mobility of urine-N in the soil, urine-N clearly poses a greater environmental threat than does faecal-N.

Numerous factors influence the fate of N in a urine patch. If conditions are favourable, plants and soil microbes will utilise the urine-N for growth or energy. If not immobilised, urine-N can be volatilised as NH_3, leached from the soil when water drains, denitrified to N_2 gas, or emitted as nitrogen oxides, such as N_2O.

Adding N to agricultural systems to sustain food production has increased N cycling, leading to greater losses of N as N_2O from agricultural systems than from natural ecosystems (Galloway *et al.*

2008). Worldwide, the production of food to sustain a growing population is generally seen as the principal reason why the concentration of atmospheric N_2O has increased over the last 250 years (Syakila and Kroeze 2011). The alteration of the nitrogen cycle by humans for food production has led to an approximate doubling of N_2O emissions above natural levels (Figure 2.1) (Galloway *et al.* 2008, Syakila and Kroeze 2011).

Figure 2.3: **Urine deposition by dairy cows grazing kale at Lincoln University's Ashley Dene farm leads to high concentrations of soil-N in small patches.**

2.4 Production of N_2O

Nitrous oxide is emitted twice in animal production systems — once when fertiliser N is applied, and again from the N that has been concentrated in animal excreta (Davidson 2009). Although a range of processes contribute N_2O to the atmosphere (Figure 2.4), animal urine deposition is the source of the majority N_2O emitted from soil in most grazed pasture settings (de Klein *et al.* 2008), therefore this review will focus on N_2O emitted from animal excretion. More than 95% of NZ's agricultural land is used for raising animal stock, either directly for meat or wool, or for dairy products, and 58% of all N_2O emitted in NZ during 2009 came from animal waste applied directly to agricultural soil (de Klein and Ledgard 2005, Ministry for the Environment 2011, 2013). In 2011, N_2O emissions from dairy cow excretion were estimated to account for approximately 45% of NZ's annual N_2O emissions from animals (Ministry for the Environment 2013). The amount of N_2O from dairy

production is likely to increase in coming years, as the number of sheep and non-dairy cattle have declined since 1990, but the number of dairy cows have nearly doubled (Ministry for the Environment 2013).

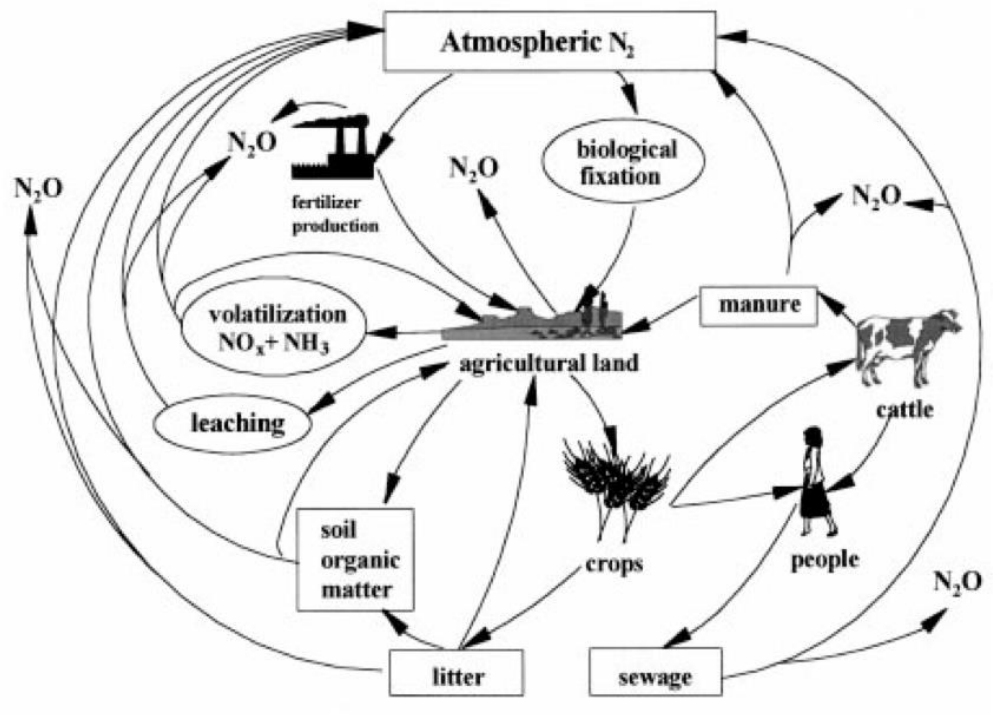

Figure 2.4: Depiction of the nitrogen cycle of agricultural soils and its relationship to N_2O production (Mosier *et al.* 1998).

Most of the N_2O that is emitted from soil originates from the bacterial processes of nitrification and denitrification (Firestone and Davidson 1989, Oenema *et al.* 1997, Mosier *et al.* 1998, Galloway *et al.* 2003). Nitrification has been defined as the biological oxidation of NH_4^+, to nitrite/nitrate (NO_2^-/NO_3^-), and denitrification defined as the biological reduction of NO_2^-/NO_3^- through to di-nitrogen (N_2). Chemical processes, such as chemodenitrification, can produce N_2O, but not in large quantities in most agricultural soil grazed by farm animals (Bremner 1997, Oenema *et al.* 1997).

Nitrous oxide is emitted as a by-product of nitrification, and an intermediate product of denitrification (Bouwman 1990, de Klein *et al.* 2008). Firestone and Davidson (1989) simplified the partitioning of N_2O emission from nitrification and denitrification with a "leaky pipe" conceptual model (Figure 2.5). In the leaky pipe model, the flow of N through the pipe represented the rate of N cycling, and the holes in the pipes represented the controlling factors that regulated the amount of NO or N_2O emitted from soil during both processes.

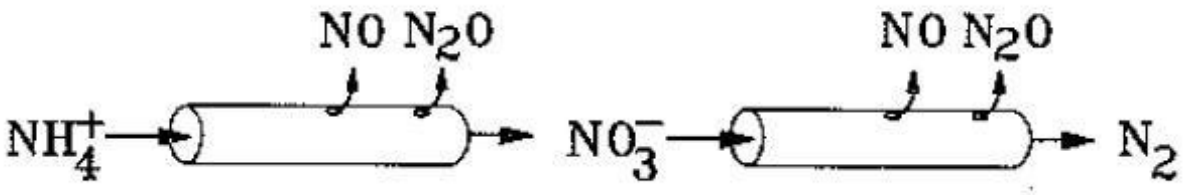

Figure 2.5: **A conceptual model of the two levels of regulation of N trace gas production via nitrification and denitrification: (a) flux of N through the process "pipes" and (b) holes in the pipes through which trace N-gases "leak" (Firestone and Davidson 1989).**

It has been suggested, however, that the "leaky pipe" model may be overly simplistic, and transformations of nitrogen within the soil can be complex and dependent on a large range of variables (Jetten 2008). There are different groups of bacteria that carry out nitrification and denitrification, autotrophic nitrifiers and heterotrophic denitrifiers (Braker and Conrad 2011), and NO_3^- is both an N source, and an electron acceptor for bacteria (Kraft *et al.* 2011). Consequently, a range of microbial processes compete for NO_3^--N in soil, including dissimilatory NO_3^- reduction to NH_4^+, anaerobic NH_4^+ oxidation, and denitrification (Kraft *et al.* 2011).

There has been a suggestion that the nitrogen cycle, which was once described as a cascade (Galloway *et al.* 2003), is more akin to a network of possible pathways for a given N molecule (Jetten 2008, Kraft *et al.* 2011). The N_2O flux from nitrification can include contributions from both NH_4^+ oxidation and sometimes, nitrifier denitrification, while N_2O fluxes from denitrification can include contributions from both denitrification, and NO_3^- ammonification (Baggs 2008, Jetten 2008). There is also some evidence to suggest N_2O may be released during dissimilatory nitrate reduction to ammonium (DNRA), which is thought to be supported in environments where NO_3^- is limiting in comparison with organic C (Kraft *et al.* 2011). However, the scale of DNRA is not fully understood (Kraft *et al.* 2011).

In N-rich agricultural soils, however, nitrification and denitrification have generally been assumed to be the primary contributors to the anthropogenic portion of N_2O production (Firestone and Davidson 1989, Bremner 1997, Oenema *et al.* 1997, Mosier *et al.* 1998, Galloway *et al.* 2003, Braker and Conrad 2011).

While it is feasible to quantify total N_2O emissions from soil, partitioning the amount of N_2O emitted solely from nitrification or denitrification is not a simple process, as N_2O emission from both

nitrification and denitrification can occur simultaneously within a soil (Bouwman 1990, Granli and Bockman 1994). Moreover, the rate of denitrification in soil is difficult to measure in the field, as the main product of denitrification, N_2, comprises 79% of the Earth's atmosphere and such high background concentrations prevent the detection of small changes in N_2 emissions (Granli and Bockman 1994, Robertson and Vitousek 2009).

2.5 Nitrification

Some organisms have been identified that can carry out heterotrophic nitrification, although it is generally accepted that autotrophic nitrification is the dominant nitrifying process in agricultural soil (Blackmer *et al.* 1980, Bremner 1997, Braker and Conrad 2011). In the first stage of nitrification, a group of micro-organisms, primarily ammonia oxidising bacteria such as *Nitrosomanas* and *Nitrospira*, convert NH_4^+ to NO_2^-. The process occurs in two co-dependent stages, where NH_4^+ is oxidized to hydroxylamine (NH_2OH) (Equation 2.1), and then NH_2OH is oxidized to NO_2^- (Equation 2.2) (Bothe *et al.* 2000).

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (2.1)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (2.2)$$

The enzyme possessed by the organisms that enables NH_3 oxidation to occur is ammonia monooxygenase (AMO), and the enzyme that enables the oxidation of NH_2OH is hydroxylamine oxioreductase (HAO) (Bothe *et al.* 2000). The AMO enzyme is coded for by the *amoA* gene. While NH_4^+ oxidizing archaea have been found to be numerically more abundant than NH_4^+ oxidising bacteria in soil (Leininger *et al.* 2006), increases in bacterial, rather than archaeal, *amoA* gene copies, have been associated with nitrification under animal urine patches, leading to the conclusion that bacteria mediate nitrification in grazed pasture systems (Di *et al.* 2009, Jia and Conrad 2009). Furthermore, in a review, Braker and Conrad (2011) found no evidence to suggest ammonia oxidizing archaea were involved in N_2O production.

Nitrous oxide is not produced directly by microorganisms during NH_4^+ oxidation, instead, some NH_2OH can be chemically decomposed to N_2O (Braker and Conrad 2011), or NO_2^- reduced to N_2O via NO in a process termed nitrifier denitrification (Figure 2.6) (Bremner 1997, Wrage *et al.* 2004, Braker and Conrad 2011). The amount of NO or N_2O produced by the chemical decomposition of NH_2OH is typically orders of magnitude lower than the amount of NO_2^- produced (Braker and Conrad 2011). Consequently, Kool *et al.* (2011) considered any N_2O produced when NH_4^+ is oxidized, to be a small portion of the total N_2O flux from agricultural soil, and dependent on whether or not NO_2^-, the final end product of NH_4^+ oxidation, is further reduced (Braker and Conrad 2011). Nitrifier denitrification is

thought to proceed in situations where there is high N, but low organic C, and low O_2 pressure (Braker and Conrad 2011). While nitrifier denitrification may contribute more N_2O than denitrification in certain conditions (Wrage *et al.* 2004), other authors (e.g. Monaghan and Barraclough 1993, Kool *et al.* 2011) suggest that denitrification is the dominant N_2O forming process occurring in soils. Supporting such a hypothesis, a review of studies found that high rates of N_2O emission coincided with anaerobic conditions associated with denitrification, rather than nitrification, leading the authors to conclude that the majority of N_2O emitted from intensive animal agriculture occurs during denitrification (de Klein and Eckard 2008).

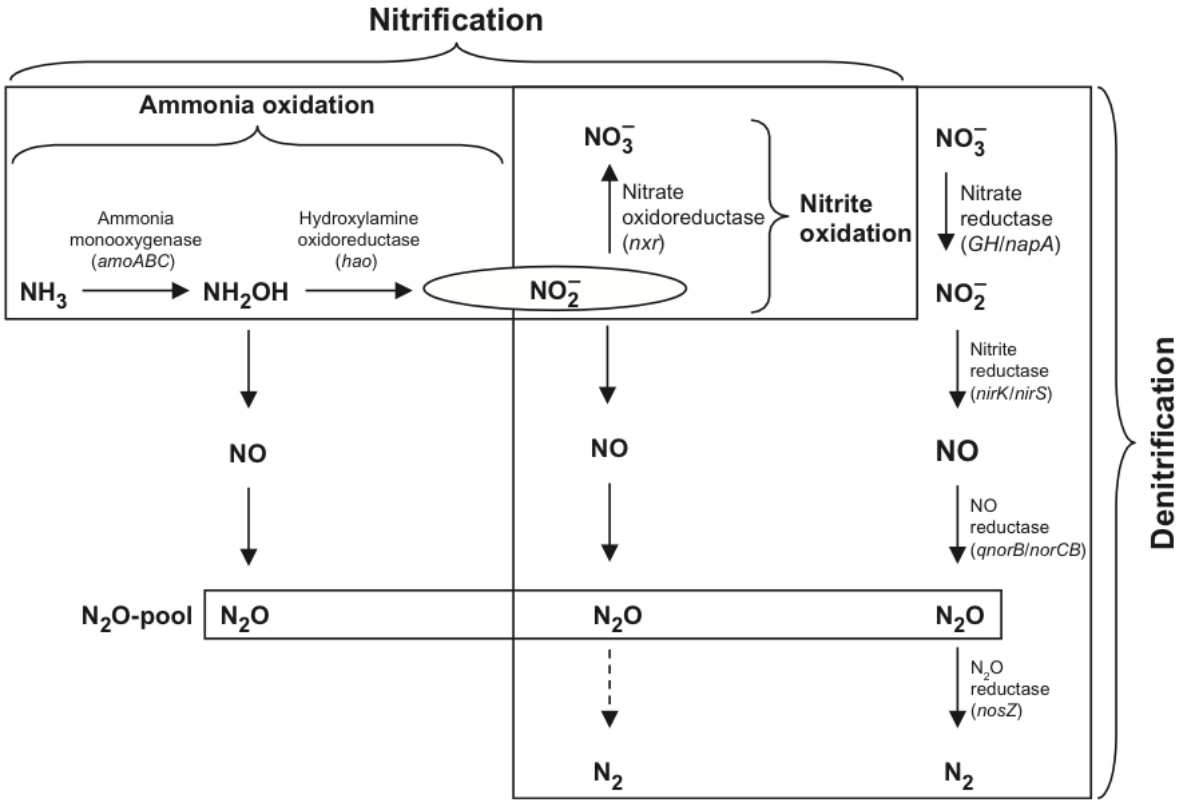

Figure 2.6: Biological reactions of the nitrogen cycle producing N_2O (Braker and Conrad 2011).

2.6 Denitrification

Denitrification has been defined as the stepwise reduction of NO_3^- through to N_2 gas (Equation 2.3) (Firestone 1982, de Klein *et al.* 2001).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (2.3)$$

Denitrification constitutes a major biological process that removes N from ecosystems (Wallenstein *et al.* 2006), however the products of denitrification are difficult to measure, and therefore the process is difficult to quantify. Furthermore, denitrifiers represent only a small portion of the total bacterial population in soil, and so are difficult to isolate and quantify (Henry *et al.* 2006).

2.7 Drivers of denitrification

Denitrification has traditionally been associated with bacteria (Firestone 1982, Bremner 1997), although recent work suggested that fungi may also be important denitrifiers who produce N_2O (Laughlin *et al.* 2009), and Sanford *et al.* (2012) noted that archaea possess denitrifying genes. Whether bacterial, fungal, or archaeal, denitrification is a facultative process and is only induced under anaerobic conditions where denitrification is used by soil organisms as an alternative to oxygen respiration (Wallenstein *et al.* 2006, Kraft *et al.* 2011).

Experiments have shown that N_2O fluxes from denitrification are highly variable both spatially and temporally (e.g. Ball *et al.* 2000b, Mathieu *et al.* 2006, Luo *et al.* 2008c, Ball *et al.* 2014), with a range of regulating factors. For example, soil pH and salinity have been found to affect the amount of N_2O emitted (Dalal *et al.* 2003), as has the quantity of soluble and readily decomposable soil carbon which is necessary for denitrification (Dalal *et al.* 2003). As NO_3^- is the substrate used by microbes during denitrification, the availability of soil and fertiliser N has been found to limit the rate of denitrification (Luo *et al.* 2000, Dalal *et al.* 2003). Consequently, organic C and N in the form of cattle slurry and green waste added to soil were found to increase N_2O emissions (Luo *et al.* 2008d, Ball *et al.* 2014).

Wallenstein *et al.* (2006) described distal and proximal environmental factors that control denitrification and denitrifying community structures. Distal factors included such things as long term climatic conditions, and soil parent material. Climatic variables such as long-term soil temperature are important, as not only does temperature influence the rate of denitrification (Dalal *et al.* 2003), it also influences the rate of other microbially driven processes that supply C and N (Saggar *et al.* 2009). Proximal factors, such as plant species, and readily available soil C and N, also strongly influence the amount of denitrification that may occur in a soil. Denitrification is much more likely to be controlled

by proximal factors which are directly impacted by soil managements and practices that influence available oxygen and situations where anaerobic conditions in soil are created.

Soil pores are the main pathway by which O_2 is diffused within soil (Figure 2.7). The size and number of soil pores are critical for controlling anerobiosis, and therefore denitrification (Renault and Stengel 1994). For instance, Ball *et al.* (1999b) found frequently cultivated soil released substantially less N_2O than un-cultivated soil, which they attributed to higher rates of denitrification due to a reduction in gas diffusivity in the un-cultivated soil. The content of soil pores is also important for denitrification, as soil water content is closely tied with the aerobic status of soil. Oxygen diffusion to aggregates will be inhibited if soil pores are blocked by water, even if a large pore network is present in soil (Renault and Stengel 1994). The effect of soil water status on denitrification can be rapid, as N_2O emissions from denitrification have been shown to increase within hours of rainfall (Ball *et al.* 2008).

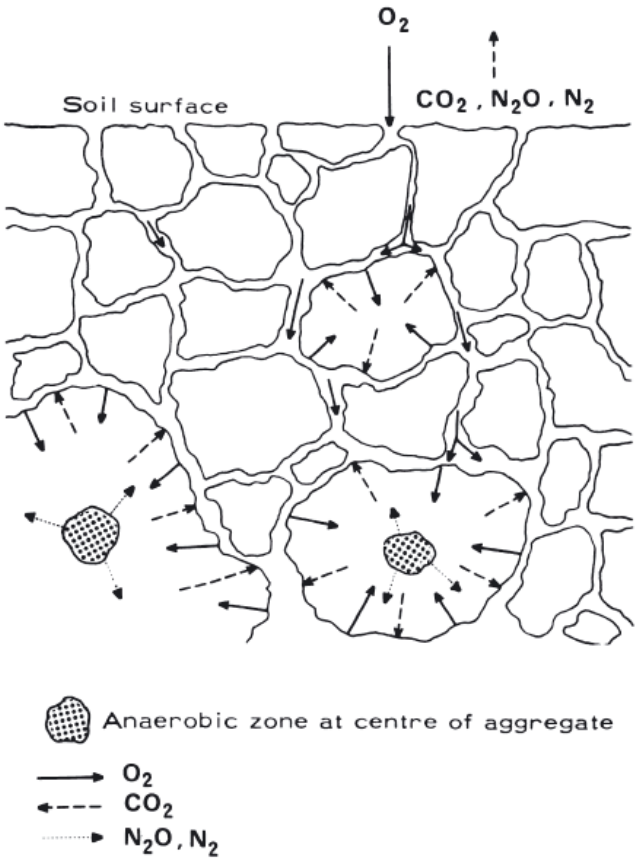

Figure 2.7: Gas movement and exchange in the soil pore system (Ball 2013).

Consequently, the proportion of soil pores occupied by water, or water-filled pore space (WFPS) is commonly associated with denitrification (Ball 2013). At soil water contents > 60% WFPS, low O_2 concentrations are thought to limit aerobic processes (Figure 2.8) (Davidson *et al.* 1991,

Bouwman 1998). In a review by Davidson *et al.* (1991), soils with a WFPS of 30-70% were deemed to be optimal for N_2O production during nitrification, and soils with WFPS above 70% conducive to N_2O emissions during denitrification. Similarly, when synthesising 20 years of research, Ball (2013) concluded that more than 75% of N_2O from agricultural soil was released when WFPS was greater than 60%. In line with such observations, (de Klein *et al.* 2003) found more urine-N was emitted as N_2O from poorly draining soils than from well-drained soils.

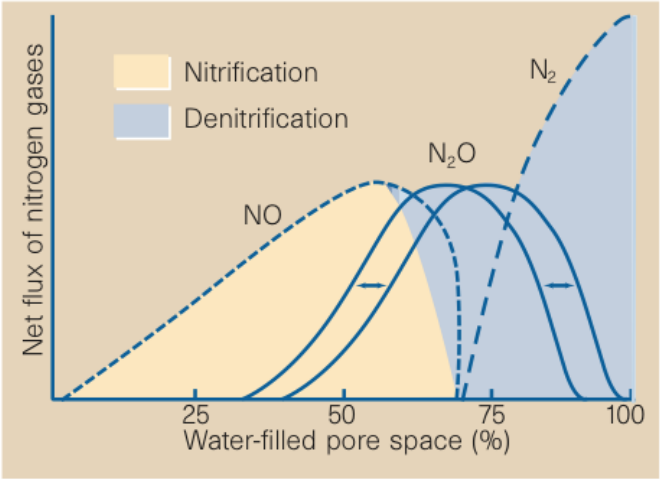

Figure 2.8: **Suggested relationship of NO, N_2O, and N_2 emissions from soil of varying water filled pores space, taken from Bouwman (1998).**

Numerous studies have linked soils that have higher water content with increased N_2O emission. For instance Luo *et al.* (2000) concluded that denitrification was limited by low soil moisture content during summer and early autumn. Ball *et al.* (1999a), Luo *et al.* (2008b), and Luo *et al.* (2008d) found N_2O emissions were only substantial when soil water content was high. Similarly. Carter (2007) concluded that at a low WFPS (about 45%), nitrification and denitrification contributed about equally to the N_2O emission flux. Ball *et al.* (1999b) showed that peak N_2O fluxes following fertilizer application occurred after rainfall. When measuring N_2O losses from urine amended soil over various seasons, Luo *et al.* (2008c) found fluxes were higher during winter and spring when the soil was wetter than in summer and autumn, even though temperatures were cooler, and microbial respiration rates slower. Likewise, Ball *et al.* (2012) noted N_2O fluxes increased dramatically in the short term following application of water to soil that had received dairy cow urine.

In grazed agricultural studies, the proportion of urine-N emitted as N_2O is often referred to as an emission-factor, denominated EF_3 in accordance with IPCC accounting methodology. The EF_3 values for animal urine have been reported as being between 0.1-3.8% (Oenema *et al.* 1997) and 0.1-4% (de

Klein *et al.* 2001), where soil aeration and drainage are cited as key drivers. Free-draining soils typically have lower EF_3 values than poorly drained soils. For instance, EF_3 values for well-drained soil in Canterbury were reported to be 0.5%, which increased to 3.7% for moderately drained soil (de Klein *et al.* 2003). Similarly, when urine was applied to a free draining sandy loam soil at different rates between 300-1000 kg N ha^{-1}, Selbie *et al.* (2014) reported EF_3 values below 0.4%. Luo *et al.* (2008c) found EF_3 values ranged from 0.02% to 1.52% when urine was applied to soil at 1,000 kg N ha^{-1}, with EF_3 values higher in winter when the soil was wetter, indicating that soil moisture content was influencing N_2O emissions.

Denitrification can be very localised, and can occur within individual soil aggregates, or can be centred around isolated sites, which probably consist of organic matter (Renault and Stengel 1994). Soil water content is important for aggregate-scale denitrification, as anaerobioisis in soil aggregates only occurs if the aggregates are nearly saturated (Renault and Stengel 1994). Soil aggregate size is important for denitrification, too, as aggregates <6 mm in size were found to be aerobic, and only aggregates >6 mm showed anaerobic conditions (Figure 2.9) (Sexstone *et al.* 1985). The rate of denitrification was not correlated with the size of anaerobic zone within aggregates, meaning other factors such C or N supply were influencing the rate of denitrification (Sexstone *et al.* 1985).

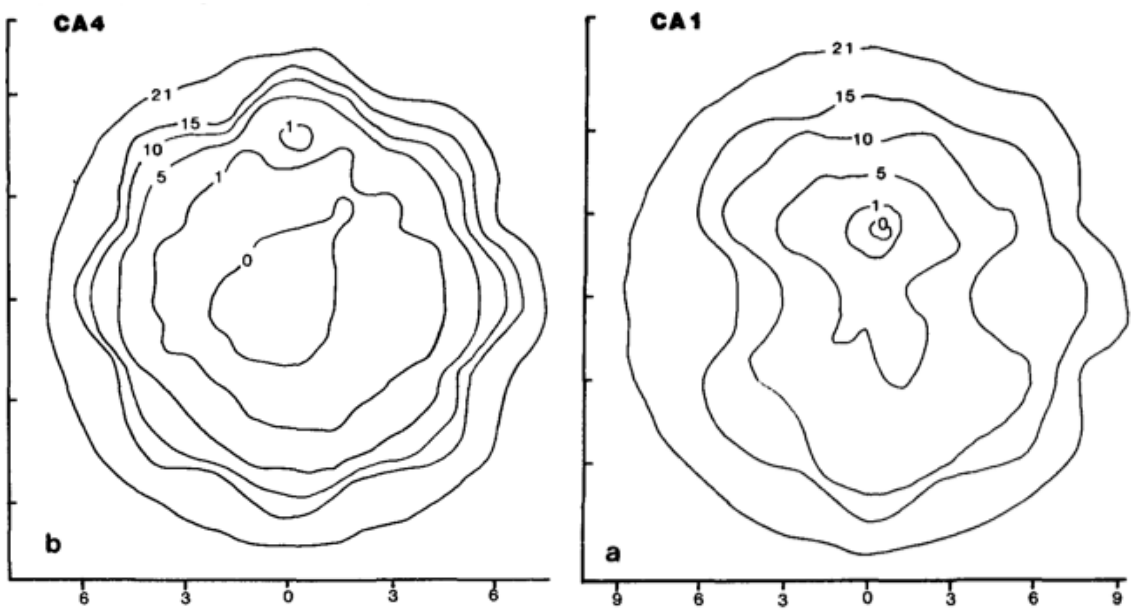

Figure 2.9: **Maps of O_2 concentration within soil aggregates. Isobars represent oxygen concentration (adapted from Sexstone *et al.* 1985).**

Conditions that increase the size of soil aggregates, or reduce the amount of oxygen that diffuses into soil will create conditions conducive to denitrification.

2.8 Animal trampling

Vehicle and animal movement are the two main processes that damage soil structure and enhance anaerobic conditions in agricultural settings. A range of terms have been used to describe soil damage in grazed systems, such as compaction, treading, trampling, poaching, or pugging (Bilotta *et al.* 2007). Animals vary in their size, weight, and level of activity, meaning it can be difficult to standardise the amount of compaction or trampling in experimental situations. Therefore, some authors have used mechanical means to simulate animal compaction or trampling damage (Di *et al.* 2001, Bhandral *et al.* 2007, van der Weerden *et al.* 2012b). For ease of reference, all animal grazing related processes that damage soil will hereby be referred to as trampling, and vehicular means will be termed compaction.

Animal trampling and soil compaction damage have been associated with a range of adverse effects (Figure 2.10), such as reduced plant growth (Di *et al.* 2001, Drewry *et al.* 2001, Nie *et al.* 2001, Pande and Yamamoto 2006, Drewry *et al.* 2008); increased soil bulk density (Di *et al.* 2001, Drewry *et al.* 2001, Herbin *et al.* 2011); decreased soil macroporosity (Di *et al.* 2001, Drewry *et al.* 2001, 2008); decreased soil air permeability (Drewry *et al.* 2001, Drewry and Paton 2005, Ball *et al.* 2012); impeded water infiltration (Drewry *et al.* 2001, Drewry and Paton 2005); and, increased soil loss from erosion (McDowell *et al.* 2003, Pande and Yamamoto 2006). All of these adverse effects can affect the amount of N_2O emitted from soil when grazed by dairy cows during winter, however previous research has focused on slow draining fine textured soil, and not free draining stony soil as is common in Canterbury.

Despite the considerable land area covered by stony soils globally, there is a paucity of published research related to stony soil (Cousin *et al.* 2014). Previous work has touched on the effect of rock fragments on soil porosity (Ma and Shao 2008); the effect of stones on soil water retention (Fies *et al.* 2002); measurement and modelling of water content in stony soils (Reinhart 1961, Spittlehouse 2000, Coppola *et al.* 2013); measurement of stony soil bulk density (Muller and Hamilton 1992, Vincent and Chadwick 1994); erosion of stony soil (Nearing *et al.* 1999); and, leaching characteristics and solute transport in stony soil (Russo 1983, Schulin *et al.* 1987, Di and Cameron 2004b, Di and Cameron 2007). Nitrous oxide emissions from grazed pasture grown in stony soil have been quantified (e.g. Di and Cameron 2002, Kelliher *et al.* 2002, Di and Cameron 2003), but not under forage grazing.

Almost no research has been undertaken to investigate the physical properties of stony soils subject to intensive grazing. Relevant research identified by the Web of Science® database when using the search key words “stony soil” and “compaction” is limited to engineering methods to measure soil compaction on construction sites (Randrup and Lichter 2001), and the effect of sheep induced

compaction on tree roots (Wairiu *et al.* 1993). Clearly, the effects of severe animal trampling on N_2O emissions, and the physical properties of stony soils, have not yet been studied.

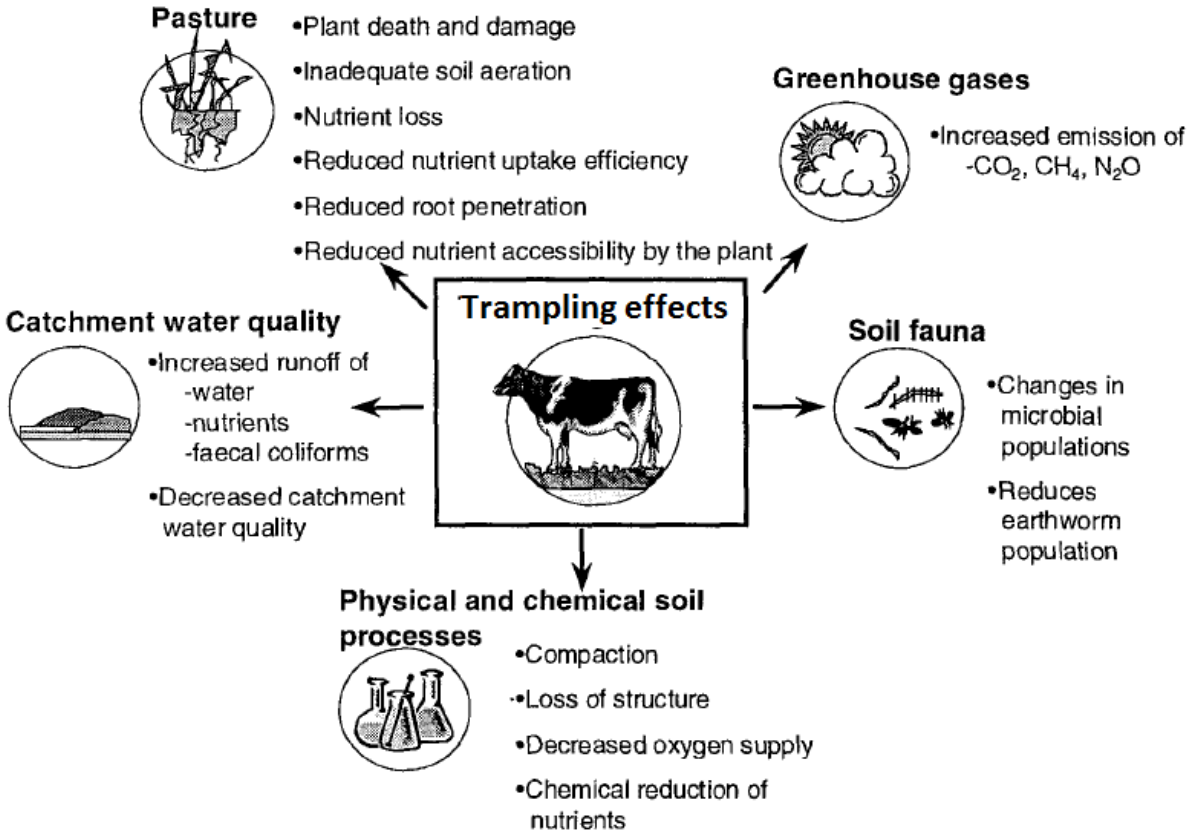

Figure 2.10: Possible on and off-site effects of animal trampling (Singleton *et al.* 2000).

2.9 Effect of animal trampling on N_2O emissions

Trampling affects the top layer of soil, and in doing so strongly influences N_2O emissions, as the majority of denitrification and N_2O production occurs in the top 5-8 cm of a soil profile (Monaghan and Barraclough 1993, Luo *et al.* 1998, Ball *et al.* 2008). Correspondingly, microbes associated with denitrification have been reported to be more abundant in topsoil layers than subsoil layers (Fischer *et al.* 2013). As a result, studies have shown that considerably more N_2O was emitted from trampled or compacted soil than from non-trampled soil (e.g. Ball *et al.* 1999a, Menneer *et al.* 2005, Simek *et al.* 2006), especially under animal urine patches (van Groenigen *et al.* 2005b, Ball *et al.* 2012).

Carran *et al.* (1995) postulated that animal trampling intensified N_2O emissions from pasture grazed in winter conditions, but was not able to quantify the contribution of trampling to the N_2O flux. Similarly, Oenema *et al.* (1997) concluded that there was very little information on the effects of animal trampling damage on N_2O emissions. A better understanding of the effects of trampling damage on

N_2O emissions has been created in the past two decades, although intensive forage grazing has received relatively little attention. The relative absence of research on the effects of animal trampling on N_2O emissions under forage grazing is concerning, as stock densities of cows grazing forage crops, can be ten times greater than stock densities when grazing pasture (Drewry and Paton 2005, Pleasants *et al.* 2007, Drewry *et al.* 2008, Moir *et al.* 2011).

In grazed pasture systems, the network of roots can provide some protection against the action of the cow hooves, limiting the amount of trampling damage (Drewry and Paton 2005, Drewry *et al.* 2008). But, the soil of forage grazing systems is more susceptible to trampling damage as a protective root network is absent. Severe trampling in grazed pasture is typically localised around gateways, water troughs, and stock camping areas (Oenema *et al.* 1997), whereas the entire paddock is trampled in forage grazing systems. If trampling occurs when the soil is wet, a common occurrence during winter, then soil is more susceptible to structural damage, and consequently higher N_2O emissions can result (Thomas *et al.* 2008, Herbin *et al.* 2011). However, the properties of the individual soil can influence the amount of trampling damage that occurs (Ball *et al.* 2000a). The degree of damage from trampling is relative to the strength of the soil, which can be influenced by the interlocking of soil particles, and the amount of sand, silt, clay, or gravels, and the plastic limit of the soil (Ball *et al.* 2000a, Bilotta *et al.* 2007). Consequently, sandy and stony soils are inherently less prone to trampling damage than finer textured soils with a higher clay content (Batey 2009). Still, stony soils are not immune to trampling damage when forage crops are grazed during winter (Figure 2.11).

Most authors who have described N_2O emissions from soil affected by trampling have concluded that the N_2O was emitted during denitrification (e.g. van Groenigen *et al.* 2005a, Ball *et al.* 2008, Thomas *et al.* 2008, Ball *et al.* 2012), which is supported by Bhandral *et al.* (2007) who found the rate of oxygen diffusion decreased in compacted soil.

Trampling compresses and reworks the soil (Betteridge *et al.* 1999, Drewry 2006, Drewry *et al.* 2008), which can lead to a collapse of soil pores (Hamamoto *et al.* 2009a), or at worst, a complete breakdown in the soil structure. Consequently, the volume of soil pores decreases when trampled, which can be characterised by an increase in the bulk density of the soil following trampling (Singleton *et al.* 2000, Di *et al.* 2001, Drewry and Paton 2005, Bhandral *et al.* 2007, Ball *et al.* 2012). A reduction in the volume and connectivity of soil pores reduces the ability of air to enter the soil. Soil pore continuity has been inversely correlated with N_2O emissions from denitrification (van der Weerden *et al.* 2012a). Measuring the permeability of soil can be a useful way to assess the soil pore network (Lipiec and Hatano 2003), and therefore the likelihood of denitrification and N_2O emission.

Figure 2.11: Trampling damage to a stony soil where forage crops have been grazed by dairy cows.

2.10 Soil air permeability

The permeability of soil to air is strongly affected by the soil structure and bulk density (Moldrup *et al.* 2001, Tyner *et al.* 2005). Accordingly, soil compaction and animal trampling have been correlated with a reduction in air permeability (AP) (Drewry *et al.* 2001, Mooney and Nipattasuk 2003, Batey 2009, Ball *et al.* 2012). The mathematical theory of soil AP is based on Darcy's flow and has been described by Kirkham (1947), with further refinement of the theory and experimental methodology by De Boodt and Kirkham (1953), Grover (1955), and Maasland and Kirkham (1955). Air permeability has since been related to gas diffusivity in soil (Moldrup *et al.* 1998, Hamamoto *et al.* 2009b), and used as a proxy for soil pore continuity (Ball 2013). Poor gas diffusivity and soil pore continuity are likely to favour denitrification and N_2O emission. Therefore, AP is a useful soil measurement that can be used to indicate the potential for denitrification in soil.

Methods of measuring soil AP have been laboratory based, using exhumed cores (e.g. Ball *et al.* 1981, Moldrup *et al.* 1998, Poulsen *et al.* 2001), or field-based, where measurements were made in-situ (e.g. Kirkham 1947, Grover 1955, Jalbert and Dane 2003, Wells *et al.* 2006). For field measurements, Ball and Schjonning (2002) suggested a surface chamber method (Figure 2.12), where pressurised air at a known flow rate was forced through the soil via a sealed chamber inserted into the soil surface, and the resultant pressure in the chamber recorded.

Laboratory methods using an exhumed core to measure soil AP can give precise results, as the area and length of soil that the air must travel through is known. In-situ AP measurements, however, require the air to pass through the known volume of soil in the soil core, and then through an unknown soil pore network (Figure 2.12). To overcome the unknown conditions at the bottom of the in-situ soil core, Grover (1955) developed a shape-factor, which was further improved by Liang *et al.* (1995) using a finite element model (ANSYS F). The ANSYS F model was further validated by Iversen *et al.* (2001) who compared previous laboratory methods using exhumed cores with in-situ field methods.

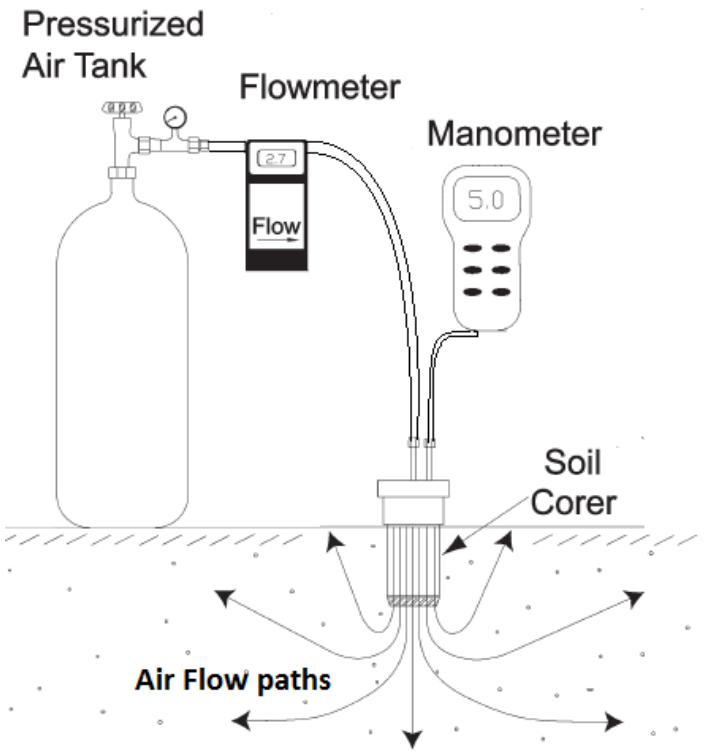

Figure 2.12: Simplified air permeameter for taking measurements in-situ (adapted from Chief *et al.* 2008b).

Since reliable methods have been developed, soil AP has been correlated with a range of variables, such as cultivation method (Mazurak and Ramig 1962); compaction (Sanchez-Giron *et al.* 1998); and, the resistance to compaction (Arthur *et al.* 2013). Air permeability has also been positively correlated with hydraulic conductivity, with AP being recommended as a time-saving alternative to saturated hydraulic conductivity (k_{sat}) measurements (Ball and Schjonning 2002, Chief *et al.* 2008a). While Moldrup *et al.* (2001) did not recommend using AP to estimate k_{sat}, others have shown a linear correlation using large numbers of samples (e.g. Loll *et al.* 1999, Iversen *et al.* 2003, Iversen *et al.* 2004,

Wells *et al.* 2006, Chief *et al.* 2008b). As the bulk of air and water in soil travels via macro pores, macroporosity has been positively correlated with AP (Drewry and Paton 2005).

Trampling has been shown to decrease macroporosity (Di *et al.* 2001, Drewry *et al.* 2004), and macroporosity has been suggested as a measure of trampling damage (Drewry 2006). However, macroporosity is time consuming to measure, which precludes the taking of a large number of samples. Measurements of AP on the other hand, are relatively quick, and can therefore encapsulate a soil's natural spatially variability (Poulsen *et al.* 2001, Wells *et al.* 2006). Rather than using macroporosity, trampling damage may possibly be assessed by taking measurements of AP.

There is very little information describing the relationship between soil AP and winter forage grazing. Only one study was identified where the effect of intensive forage grazing on soil AP was described. In the study, in line with expectations, a reduction in macroporosity, k_{sat}, and AP was observed in a fine textured Pallic soil where a winter forage crop had been grazed (Drewry and Paton 2005). Similar observations have not been reported for stony soil, which is likely to behave differently under forage grazing than a fine textured soil.

Nitrification has been linked with soil gas diffusivity and AP (Schjonning *et al.* 2003), but the relationship between soil AP and the abundance of microbes possessing denitrifying genes has not yet been reported. Furthermore, a reduction in gas diffusivity has been shown to enable N_2O consumption in soil, but gas diffusivity is still major source of uncertainty in soil process models (Maier and Schack-Kirchner 2014). As gas diffusivity and AP have been correlated, AP could also help with estimates of N_2O consumption in soil.

2.11 N_2:N_2O ratio

Denitrification produces gaseous NO, N_2O and N_2, however the mechanisms by which soil factors control the ratios of NO, N_2O and N_2 are not well understood (Saggar *et al.* 2009). Emissions of NO were found to be about 1% of N_2O emissions following urine application to pasture grown in fine-textured clay (Lovell and Jarvis 1996). Some studies suggest N_2O is reduced to N_2 within a matter of days, and N_2 emissions are generally greater than N_2O emission (Hamonts *et al.* 2013), however a variety of factors regulate the activity of the N_2O reductase enzyme within soil organisms. There has been a suggestion that because the activation energy to reduce N_2O to N_2 is higher than the activation energy to create N_2O, proportionally more N_2O might be emitted in lower temperature environments (Saggar *et al.* 2009).

Soil moisture content can affect the N_2O:N_2 ratio. Ruser *et al.* (2006) reported that N_2O emissions from denitrification from soil were greatest when the WFPS was greater than 70%, and N_2 was only produced when WFPS was greater than 90%. An increase in the N_2O:N_2 ratio (i.e.

proportionally more N_2O) has been correlated with decreasing soil pH (Firestone *et al.* 1980, Simek and Cooper 2002, Saggar *et al.* 2009), which supports the work of Yu *et al.* (2014) who found N_2O reductase gene abundance in soil was positively correlated with soil pH. In groundwater samples, N_2 was reported to be positively correlated with dissolved organic carbon (DOC) and negatively correlated with dissolved oxygen (DO) (Barrett *et al.* 2013).

Drying and wetting cycles in soil may mineralise N, which can then be denitrified to N_2 or N_2O (Ruser *et al.* 2006, Beare *et al.* 2009), but the oxic/anoxic phases during drying and wetting may inhibit N_2 production, since the fluctuating O_2 concentration inhibits the N_2O reductase enzyme (Saggar *et al.* 2009). Likewise, Firestone *et al.* (1980) concluded that a higher proportion of N_2O was produced when O_2 and NO_2^- concentrations in soil were higher, possibly because microbes obtained more energy from reducing NO than they did from reducing N_2O, and therefore the N_2O reductase enzyme was more inhibited by O_2 concentration than NO_3^-, NO_2^-, or NO reductase enzymes (Saggar *et al.* 2009). Similarly, Blackmer and Bremner (1978), and Saggar *et al.* (2009) reported that high concentrations of NO_3^- inhibited the ability of soil microorganisms to reduce N_2O to N_2.

In addition to the effects of environmental controls on the N_2O:N_2 ratio, the diverse soil microbial population may also play a part in determining the proportion of N_2O is emitted during denitrification. Some denitrifiers exist where NO_2^- or N_2O are the sole respiratory electron receptor (Bothe *et al.* 2000, Philippot *et al.* 2011), therefore, N_2 is not produced by all denitrifiers. Analysis of known denitrifying genomes discovered about 1/3 lacked the N_2O reductase gene (Philippot *et al.* 2011). However, if situations are favourable, N_2O can be consumed by other organisms within the soil (Chapuis-Lardy *et al.* 2007). In one study, N_2O produced by bacteria lacking the N_2O reductase gene appeared to be consumed by other denitrifiers, with the soil effectively acting as a sink for N_2O, which lead to the suggestion that the N_2O uptake ability of the soil is more important for N_2O emissions than the proportion of denitrifiers lacking the N_2O reductase gene (Philippot *et al.* 2011). Furthermore, Some N_2O reductase bearing microbes have been discovered which do not possess other denitrifying genes, so they must rely on other organisms to supply N_2O if they use N_2O for respiration (Sanford *et al.* 2012). If gas diffusion is restricted, such as in compacted or trampled soil with a damaged pore network, a large amount of the N_2O produced could be consumed within the soil, and not emitted (Chapuis-Lardy *et al.* 2007, Fowler *et al.* 2009).

2.12 Methods of measuring N_2O emissions and denitrification

Collecting and measuring all the N_2O emitted from a grazed field is a near impossible task. However, by using lysimeters or plots to simulate urine and non-urine affected portions of a field, it is possible to scale-up emissions to estimate field N losses using computer simulations or GIS mapping

(Lilly *et al.* 2003, Lilburne *et al.* 2012). The use of closed chambers such as those described by Hutchinson and Mosier (1981) can be used in conjunction with lysimeters or plots, providing a method considered to be reasonably accurate (Oenema *et al.* 1997). A commonly used chamber methodology was described in detail in de Klein *et al.* (2003), where analysis showed a linear increase in N_2O concentration over time from a field where cow urine was applied. While N_2O emissions from soil can vary spatially and temporally, comparisons with automated chambers showed that N_2O concentrations in samples taken in the middle of the day were representative of the average for that day (de Klein *et al.* 2003).

There are few data describing rates of denitrification in stony soil, possibly because the stones prevent undisturbed cores from being exhumed, which eliminates the use of laboratory methods that require undisturbed cores. Micro-meteorological methods have been used to assess N_2O emission from grazed stony soil in Canterbury. However, the method required a large area to be effective, and high spatial variability led the authors to recommend the use of chambers instead (Kelliher *et al.* 2002). Chambers can be used to collect samples for both N_2 and N_2O, but it is not possible to determine the source of those emissions without additional measures.

Stable isotopes of N, particularly ^{15}N, have been used to follow the fate of N applied to soil (Laughlin and Stevens 2003). Clough *et al.* (2013b) reviewed the use of ^{15}N as a means to trace urine-N in soil, and the reader is referred there for a complete description of the technique. By enriching urine with ^{15}N urea, the proportion of urine-derived N_2O emitted from soil can be calculated, as can the N_2 flux (Clough *et al.* 2013b). Clough *et al.* (2013b) also suggested using stable ^{18}O isotopes, and ^{18}O-labelled NO_3^- to help differentiate between N_2O emissions from nitrification and denitrification. With the high background concentration of N_2 in the atmosphere (about 78%), ^{15}N concentrations in N_2 can be difficult to measure if the ^{15}N enrichment is insufficient. However, Yang *et al.* (2014) used gas chromatography to purify gas samples before isotope ratio mass spectrometry to increase precision.

One method of determining the likelihood of denitrification in soil involves assessing the denitrifying enzyme activity (DEA), or potential denitrification, in a laboratory. The methodology was described by Luo *et al.* (1996), where unlimited C and N sources were added to water-saturated soils, then flooded with C_2H_2 to prevent N_2 production. By saturating the soil with water, nitrification was said to be inhibited, and by preventing N_2 production, only N_2O was emitted. Therefore, the amount of N_2O emitted, as measured by gas chromatography, represented the amount of N denitrified.

Further methods have used nitrification inhibitors other than C_2H_2 to differentiate between nitrification and denitrification (Koops *et al.* 1997), although by limiting nitrification so only denitrification occurs, the substrate for denitrification, NO_3^-, is not produced, which could lead to an under-estimation of the rate of denitrification (Conrad 1996, Groffman *et al.* 2006, Fowler *et al.* 2009).

Therefore, nitrification inhibiting methods such as DEA, can only give a 'snap shot' picture of the potential denitrification in a soil.

Advances in genetic technologies have allowed researchers to study, and begin mapping, the genetic makeup of a soil's microbial population. One approach involves targeting the functional genes that code for enzymes involved in key N transformations within organisms. Distinct enzymes facilitate various processes, and therefore identifying the abundance of the genes that code for these enzymes can give an insight into the processes that may be occurring in soil. The abundance and activity of the various genes present in the soil can be correlated with the substrate availability (NH_4^+ and NO_3^-), and the rate of N_2O emitted, and therefore point to the source of the N_2O. At the time of writing, no authors had described the way in which animal trampling influenced the denitrifying microbial population in soil.

2.13 Denitrifying soil microbes

The biological process of denitrification has been comprehensively reviewed by Firestone (1982), with further reviews from Zumft (1997), Philippot (2002), Braker and Conrad (2011), and Saggar *et al.* (2013). The denitrifying microbial community is diverse, and many of the bacterial species have not yet been cultured in the laboratory, which prohibits the use of culture-based techniques in denitrification studies (Groffman *et al.* 2006). Such problems can be overcome by using techniques that target common functional marker genes shared by bacterial species, which can be used to gauge the relative size of the denitrifying community in a range of samples (Figure 2.13). The functional marker genes code for enzymes that catalyse each step in the denitrification pathway (Wallenstein *et al.* 2006, Braker and Conrad 2011). Genes that code for the NO_3^- reductase Nar enzyme include *nar* and *nap* and their subunits, while two evolutionarily and physiologically distinct types of NO_2^- reductase Nir enzymes and are coded for by *nirS* and *nirK* genes. The NO reductase Nor enzyme catalyses the production of N_2O, and is coded for by *nor* genes, and the Nos enzyme responsible for the final step of denitrification – reduction of N_2O to N_2 – is coded for by the *nos* genes (Figure 2.13).

NO_3^-	→	NO_2^-	→	NO	→	N_2O	→	N_2
	Nar		**Nir**		**Nor**		**Nos**	
	nar/nap		*nirS/nirK*		*nor*		*nos*	

Figure 2.13: Common enzymes (underlined) and genes (italicised) associated with denitrification (Philippot 2002).

It is important to select appropriate target genes in denitrification studies. Nitric oxide is a radical that will bind with many non-specific molecules and can be toxic to organisms. Consequently, organisms that are capable of producing NO – which can include nitrifiers – contain the NO reducing Nor enzyme, and associated *nor* genes. Additionally, some non-denitrifying organisms possess the NO_3^- reductase genes *nar* and *nap* genes. Consequently, focussing solely on the *nap*, *nar*, and *nor* genes is not ideal for denitrification studies, and workers typically concentrate on other denitrifying genes (Wallenstein *et al.* 2006, Saggar *et al.* 2009, Kraft *et al.* 2011). During denitrification, the reduction of NO_2^- to NO is the first stage where gas is produced, and so the *nir* genes are commonly targeted in N_2O emission studies (Bothe *et al.* 2000, Groffman *et al.* 2006). However, Braker and Conrad (2011) suggested that exclusively targeting the *nir* genes may detect nitrifiers and denitrifiers who carry out nitrifier denitrification and heterotrophic denitrification, as well as fungal denitrifying genes. Similarly, *nirK* gene homologues have reportedly been detected in ammonia-oxidising archaea (Bartossek *et al.* 2010). In contrast, the final stage of denitrification, N_2O reduction, is exclusive to denitrifiers and requires the Nos enzyme, which is coded for by *nos* genes. Therefore, many workers target the *nir* and *nos* genes together in denitrification studies.

Methods that target the functional genes in soil begin by removing DNA from the organism's cells, after which time a range of techniques can be used depending on the required outcome (Figure 2.14). Many commonly used methods use polymerase chain reaction (PCR) technology, where selective fragments of DNA are amplified. The structure and diversity of denitrifying communities can be assessed following PCR by using additional techniques such as denaturing gel gradient electrophoresis (DGGE), or terminal-restriction fragment length polymorphism (TRFLP) (Wallenstein *et al.* 2006). For instance, using DGGE, Enwall *et al.* (2005) found that different long term fertiliser strategies changed the denitrifying community structure, or the soil's DNA 'fingerprint'.

However, if the objective is to assess denitrifying conditions in soil, it can be more useful to quantify the abundance of microbes containing the target genes, rather than the diversity of the microbial community. To that end, a modification to the PCR technique termed quantitative-PCR (pPCR) has been developed which allows researchers to quantify the abundance of target genes in soil samples (Wallenstein *et al.* 2006, Correa-Galeote *et al.* 2013).

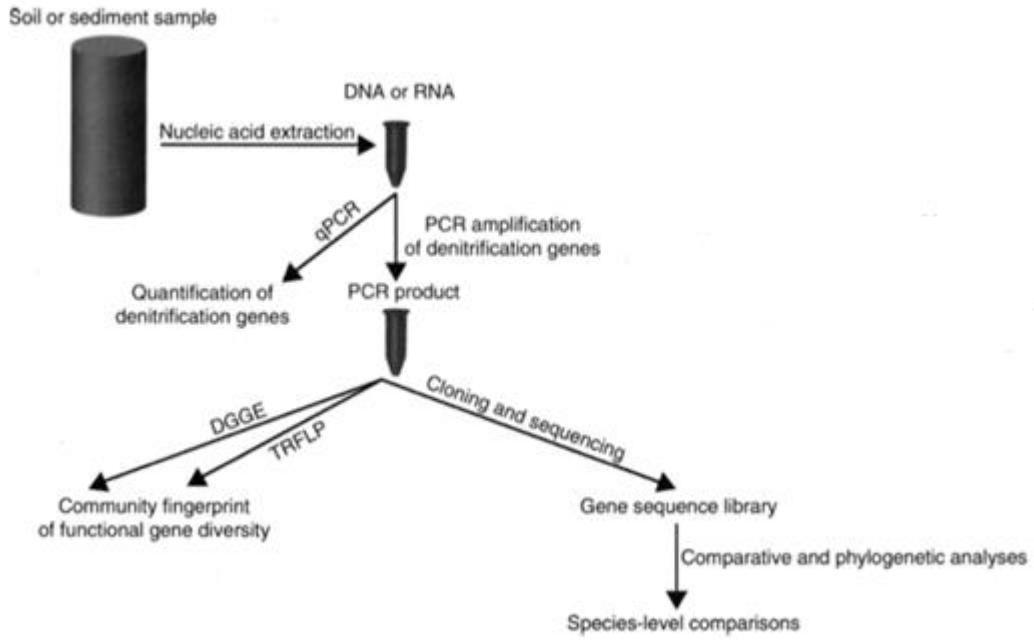

Figure 2.14: **Alternative molecular techniques for examining denitrifier communities following extraction of nucleic acids from environmental samples. Products from the polymerase chain reaction technique can be used to assess the diversity or structure of the denitrifying community using denaturing gel gradient electrophoresis (DGGE) or terminal-restriction fragment length polymorphism (TRFLP). A modification to the PCR method (qPCR) can be used to quantify the relative abundance of denitrifying genes (modified from Wallenstein *et al.* 2006).**

2.14 Techniques for microbial population quantification

Denitrifying organisms are a phylogenically diverse group including bacteria, fungi, and archaea (Wallenstein *et al.* 2006, Sanford *et al.* 2012), and considerable work has been undertaken to classify and map such groups (Dandie *et al.* 2007, Baggs 2008). Even though the complete range of denitrifying microorganisms has not yet been classified (Green *et al.* 2010), PCR has been used successfully to quantify the abundance of denitrifying genes in a range of environmental situations.

The extensive range of environmental conditions where denitrifying genes have been detected includes ocean, estuarine, and freshwater sediments (Braker *et al.* 2000, Lopez-Gutierrez *et al.* 2004, Smith *et al.* 2007, Reyna *et al.* 2010); ocean water (Castro-Gonzalez *et al.* 2005, Oakley *et al.* 2007, Jayakumar *et al.* 2009); groundwater (Yan *et al.* 2003, Green *et al.* 2010, Barrett *et al.* 2013); sewage sludge (Hallin and Lindgren 1999, Geets *et al.* 2007, Nittami *et al.* 2009); rice paddy soil (Yoshida *et al.* 2009, Chen *et al.* 2010, Yoshida *et al.* 2010); forest soil (Levy-Booth and Winder 2010, Theerachat *et al.* 2011, Yu *et al.* 2014); and, agricultural soil (Dandie *et al.* 2011, Di *et al.* 2014, Yu *et al.* 2014).

The PCR technique uses synthetic DNA molecules, termed primers, which have a sequence matching part of the target gene DNA (Wallenstein and Vilgalys 2005). Primers have successfully been

developed for all known denitrification genes, for instance, primers for *narG* were developed using real-time PCR (Lopez-Gutierrez *et al.* 2004), while *nirS* and *nirK* primers have been produced by Braker *et al.* (1998). Primers for *nosZ* have been developed by Kloos *et al.* (2001) and verified by Henry *et al.* (2006), who detected *nosZ* genes in soil samples using qPCR.

While considerable work has already been undertaken to develop PCR primer sets, not all primer sets are appropriate in all situations, for instance Michotey *et al.* (2000) developed two PCR primer sets for the *nirS* gene, but only one set was found to be useful for quantification. And while Throback *et al.* (2004) developed improved PCR primers for *nirS* and *nosZ,* other authors have reported more success using primers revised by Henry *et al.* (2006). Further challenges arise as qPCR has been used successfully to quantify *nirK* gene copy abundance in five different soils (Henry *et al.* 2004), however not all *nirK* are identical, and common primers reportedly do not capture part of class II *nirK* genes (Nittami *et al.* 2009, Braker and Conrad 2011). Likewise, some atypical denitrifiers that possess *nosZ,* but no other denitrifying genes, may not be detected using common primer sets (Sanford *et al.* 2012). Despite the possible shortfalls, the use of PCR to quantify the abundance of denitrifying microorganisms continues to improve. For instance, recent work by Jones *et al.* (2013) identified a previously undetected *nosZ* lineage termed clade-II, which led to the creation of a new set of primers capable of quantifying the abundance of *nosZ* clade-II genes. The relative abundance in different environments was found to be similar between *nosZ*-I and *nosZ*-II genes (Jones *et al.* 2013), and it has been suggested that the *nosZ*-II gene is more important for mediating N_2O consumption in soil than the previously identified *nosZ*-I gene (Jones *et al.* 2014).

Comparisons between microbial techniques and more traditional methods of measuring denitrification have had mixed results. For instance, potential denitrification was positively correlated with *norB*, but not *narG*, *nirK*, or *nosZ* genes (Yu *et al.* 2014). Similarly, DEA was not correlated with denitrifying *nirK*, *nirS*, or *nosZ* abundance (Attard *et al.* 2011, Dandie *et al.* 2011, Le Roux *et al.* 2013).

Within soil, correlations between the abundance or diversity of denitrifying genes have been observed with a range of variables. The variables reported to favour denitrifying genes support previous work where denitrification has been said to occur, and include the age of the soil (Kandeler *et al.* 2006, Brankatschk *et al.* 2011); soil pH (Dandie *et al.* 2011, Hamonts *et al.* 2013, Yu *et al.* 2014); carbon availability (Henry *et al.* 2004, Levy-Booth and Winder 2010, Fischer *et al.* 2013); nitrogen availability (Levy-Booth and Winder 2010, Xue *et al.* 2013); the addition of compost or organic matter (Barta *et al.* 2010, Maeda *et al.* 2010, Paranychianakis *et al.* 2013); and, soil chemistry changes in the plant rhizosphere (Mounier *et al.* 2004, Babic *et al.* 2008, Hamonts *et al.* 2013). In line with authors who have correlated denitrification with increases in a soil's water filled pore space, the abundance of denitrifying genes has been correlated with increases in saturated soil, such as rice paddies, or riparian buffer soil (Chen *et al.* 2010, Yoshida *et al.* 2010, Dandie *et al.* 2011, Wu *et al.* 2012). Likewise, Tatti *et*

al. (2013) observed little change in denitrifying *nirS*, *nirK*, and *nosZ* gene abundance when soil was stored in oxic microcosms.

Most studies quantifying denitrifying genes have been laboratory-based, where environmental conditions were closely controlled. For example, in a laboratory study, Hamonts *et al.* (2013) examined the effect of two different soil bulk densities on the abundance of denitrifying genes, but no long-term trends were detected during the 35 day study period. In a field study, (Chroňáková *et al.* 2009) reported an increase in *nirS*, *nirK*, and *nosZ* genes in soil that had been impacted by cattle in overwintering, which they attributed to an increase in the supply of C and N. To date, no authors have described the influence of animal trampling of soil on denitrifying gene abundance, especially under field conditions.

2.15 Nitrous oxide emission mitigation options

Several reviews have discussed N_2O emission mitigation options from grazed agriculture (e.g. Oenema *et al.* 1997, Dalal *et al.* 2003, de Klein and Ledgard 2005, de Klein and Eckard 2008, Eckard *et al.* 2010, Luo *et al.* 2010). Most reviews suggested increasing the N efficiency of the animal so less N was excreted. Opportunities to increase N efficiency relied on increasing animal production for a given amount of feed, or reducing the amount of N ingested by the animals (e.g. Luo *et al.* 2008a). Other options included increasing the proportion of N excreted in faeces rather than urine, or diluting the animal's urine by providing a diuretic such as salt (NaCl) (de Klein and Eckard 2008, Spek *et al.* 2012). Furthermore, the dry matter content of animal feed can influence the amount of water intake, and subsequently affect the amount and N concentration of urine voided by dairy cows (Khelil-Arfa *et al.* 2012).

On-farm management methods identified to mitigate N_2O emissions generally followed industry best-practice guidelines, such as avoiding wet season grazing, irrigating efficiently, and managing soil water content through surface or subsurface drainage (Oenema *et al.* 1997, de Klein and Ledgard 2005, Monteny *et al.* 2006, de Klein and Eckard 2008, Eckard *et al.* 2010).

Adjusting grazing management by utilising a stand-off pad may also be useful for reducing N_2O emissions from winter grazed systems. Not only is the potential for trampling-induced denitrification reduced when using a stand-off pad, but less N is excreted onto the soil as well (Luo *et al.* 2008b). Considerably less N_2O was emitted from a stand-off pad than from pasture for the same given urine-N input (Luo and Saggar 2008).

Nitrification inhibitors have also been recommended as a cost-effective means to reduce the amount of N_2O emitted from grazed soil (e.g. Monteny *et al.* 2006, de Klein and Eckard 2008, Doole

2014). Dicyandiamide (DCD) is a nitrification inhibitor that has received considerable research interest as it not only can reduce N_2O emissions, but also reduce NO_3^- leaching (Di and Cameron 2002, Monaghan *et al.* 2013, Cameron *et al.* 2014).

2.15.1 DCD

Dicyandiamide (DCD, $C_2H_4N_4$) inhibits nitrification by binding to the receptor site for the ammonia monooxygenase enzyme, preventing microorganisms from carrying out nitrification. By slowing the process of nitrification, DCD reduces soil NO_3^- content, reducing the substrate available for denitrification, and therefore reducing the amount of N_2O emitted via denitrification (de Klein and van Logtestijn 1994).

Weiske *et al.* (2001), Smith *et al.* (2008a), and Singh *et al.* (2009) have shown DCD to be a powerful tool for reducing N_2O emissions from grazed agricultural systems. When reviewing published research, Clough *et al.* (2007) found that, on average, DCD reduced net N_2O emissions by 72%. Luo *et al.* (2013b) also reported that using DCD together with a winter stand-off pad was more effective at reducing N_2O emissions than using either option alone.

Evidence suggests DCD would be an ideal mitigation strategy to use in a winter forage grazing system. Recently, the N_2O emission mitigation efficiency of DCD was found to increase with increasing urine-N load (Selbie *et al.* 2014), and Ball *et al.* (2012) found the effectiveness of DCD was not hampered by trampling. In addition, the rate of degradation of DCD is temperature dependent, and therefore DCD is most effective during the cooler winter months when soil temperatures are below 10 °C (Di and Cameron 2004a, Kelliher *et al.* 2008). The high urine-N deposition rate, together with the cool temperatures experienced during winter, would suggest that DCD would be a suitable option for mitigating N_2O emissions from winter forage grazing. In contrast to the large number of studies where DCD has been applied to grazed pasture, there is limited research on the effect of DCD to reduce N_2O emissions from grazed winter forage systems.

In grazed winter forage systems, Smith *et al.* (2008b), van der Weerden *et al.* (2012b), and Monaghan *et al.* (2013) have shown DCD to be effective in reducing N_2O emissions from fine-textured Pallic soils, but similar research on free draining stony soil in Canterbury has not yet been reported. As well as the 25% reduction in N_2O emissions from the forage crop, Monaghan *et al.* (2013) noted that the application of DCD conserved soil-N during winter, which resulted in increased yields from the pasture planted the following season.

In addition to DCD, several other nitrification inhibiting substances exist, such as karanjin, nitrapyin, and 3,4-dimethylpyrazole phosphate (DMPP) (Bedard and Knowles 1989, Majumdar 2002,

Cameron *et al.* 2013). Besides nitrification inhibitors, a large body of evidence is growing that advocates the use of charcoal to supress N_2O emissions from agricultural soil.

2.15.2 Biochar

The Earth's soil contains more than three times as much carbon as does the atmosphere and all plants combined (Schmidt *et al.* 2011). Consequently, many authors have proposed humans manipulate the carbon cycle and use soil as a sink for atmospheric CO_2, and in doing so, reduce the threat of global climate change (Lehmann 2007b, a, Steinbeiss *et al.* 2009, Clough and Condron 2010, Sohi *et al.* 2010, Woolf *et al.* 2010). When organic matter is heated in the absence of oxygen, charcoal is formed. The term biochar has been associated with charcoal used as a soil amendment for agronomical benefit (Lehmann and Joseph 2009). In addition to carbon sequestration, other benefits have been noted when biochar is worked into soil, including improved soil nutrient retention through increased cation exchange capacity (CEC) (Lee *et al.* 2010, Van Zwieten *et al.* 2010a, Peng *et al.* 2011); increased water holding capacity (Jeffery *et al.* 2011, Case *et al.* 2012, Karer *et al.* 2013); the creation of a potential refuge for soil biota (Steinbeiss *et al.* 2009, Lehmann *et al.* 2011); and a decrease in soil bulk density/increase in porosity (Chan *et al.* 2007, Atkinson *et al.* 2010, Case *et al.* 2012). Moreover, N adsorbed by the addition of biochar to soil has been shown to be plant available, suggesting biochar has the potential to increase plant growth (Taghizadeh-Toosi *et al.* 2012, Zheng *et al.* 2013).

While several studies have had success using biochar as a soil amendment to suppress N_2O emissions (e.g. Atkinson *et al.* 2010, Singh *et al.* 2010, Van Zwieten *et al.* 2010b, Taghizadeh-Toosi *et al.* 2011), other studies question the effectiveness of biochar and its ability to reduce N_2O emissions (e.g. Clough *et al.* 2010, Scheer *et al.* 2011, Ameloot *et al.* 2013, Angst *et al.* 2014). The mechanisms by which biochar suppresses N_2O emissions is the subject of considerable ongoing research. Biochar is thought to produce physical, chemical, and biological changes within soil, leading to various alterations of the N cycle (Lehmann and Joseph 2009). However, in recent reviews, both Clough *et al.* (2013a), and Cayuela *et al.* (2014) described the controversy and lack of understanding surrounding biochar's ability to influence N_2O emissions. In light of such conclusions, the effect of biochar on denitrification is not well known. Improved soil aeration may reduce the incidence of denitrification (Case *et al.* 2012), but conversely, Cayuela *et al.* (2013) suggested the mild liming effect when biochar was added to soil may combine with an "electron shuttle" effect that assists in transferring electrons to denitrifying microorganisms, which may enhance the reduction of N_2O to N_2. On the contrary, the addition of C may facilitate denitrification in soil with a low C content and may promote N_2O production.

Biochar has been shown to remove NH_4^+ from solution and dairy effluent (Ding *et al.* 2010, Hale *et al.* 2013, Hollister *et al.* 2013, Sarkhot *et al.* 2013). The application of DCD to urine affected soil increases the amount of time that the urine remains in the NH_4^+ form. When applied together, DCD and biochar may have a complementary effect, retaining urine-N in the soil and reducing the potential for N_2O emission. However, the effectiveness of using a combination of biochar and DCD as a method of reducing N_2O emissions has not been tested.

2.16 Literature summary

Nitrous oxide has been identified as one of the fourth most important anthropogenically produced greenhouse gases. Robertson and Tiedje (1987) concluded that there was little agreement on the primary source of N_2O from soil. However, this literature review has revealed that in experiments during the past three decades, N_2O emissions from soil where conditions favour denitrification were consistently higher than from soil where denitrification was unlikely, and that most N_2O from agricultural soil is emitted during denitrification.

In NZ's GHG Inventory 1990-2009, the Ministry for the Environment concluded that "*Forage brassicas have been identified as an important crop in New Zealand but activity data is currently inadequate to be able to carry out emission calculations."* (Ministry for the Environment 2011, p 138). Winter grazing of forage crops by dairy cows combines high concentrations of animal urine with soil compaction, both of which have been shown to independently increase N_2O emission rates. Therefore, WDFS provide the perfect conditions for large N_2O losses, and an ideal environment for targeted strategies to reduce N_2O losses, which could help to reduce NZ's overall GHG emissions.

Several options to mitigate N_2O emissions were identified, including DCD, a nitrification inhibitor which increases soil NH_4^+ concentration, and biochar, a substance capable of adsorbing soil NH_4^+. Neither of these mitigation options have been trialed in a forage system on coarse textured stony soil. Furthermore, the complimentary properties of DCD and biochar in combination have not yet been tested.

Despite the commonly cited conclusion that animal trampling increases the rate of denitrification in soil, no authors have described the effect of animal trampling on the denitrifying microbial population – the organisms largely responsible for N_2O emissions. And despite the common occurrence of stony soils globally, there has been little research including stony soils when compared with research featuring finer textured soils.

There is therefore an urgent need for new knowledge and a better understanding of denitrification and N_2O emissions from forage systems grazed by dairy cows during winter.

Chapter Three – Experimental design

3.1 Key Hypothesis

A review of published literature (Chapter Two) identified a serious absence of knowledge on the occurrence of denitrification and the amount of nitrous oxide emitted from forage crops intensively grazed by dairy cows in winter, or ways to mitigate those emissions. Therefore, the key hypothesis defining the research for this thesis was:

The potentially high substrate availability (urine-N), and animal trampling effects in a winter dairy forage system, will favour denitrifier activity and high rates of N_2O production via denitrification, which can be reduced by the application of DCD and biochar.

3.2 Objectives

To test the key hypothesis, four research objectives, and six research questions were established.

3.2.1 Research objectives

- Quantify the amount of N_2O emitted from a free-draining stony soil that is typically used in Canterbury for the winter dairy forage system;
- Determine the effectiveness of the nitrification inhibitor, DCD, and biochar, in reducing the amount of N_2O emitted from a winter dairy forage system;
- Improve knowledge and understanding on the effect of cow urine, and DCD, on the abundance and activity of denitrifying bacteria in winter dairy forage system soils, and of their relationship to the N_2O emission flux, and;
- Investigate the effects of animal trampling on soil physical conditions, soil microbial populations, and N_2O emissions in a winter dairy forage system.

3.2.2 Research questions

- How much N_2O is emitted from a stony Canterbury soil subjected to animal trampling with & without the addition of animal urine?
- How effective is DCD and/or biochar at reducing N_2O emissions from a winter dairy forage system soil?

- To what extent will DCD and/or biochar affect the microbial denitrifier populations in a winter dairy forage system soil?
- Will animal treading induce changes in the air permeability, water-filled porosity, and bulk density of the soil within a winter dairy forage system?
- Will animal trampling induce changes in the microbial denitrifier population, and N_2O emissions from denitrification, when compared to non-trampled treatments?

3.3 Experimental chapters

This thesis features four experimental chapters to meet the research objectives, and answer the research questions. Three field-based experiments (Chapters Four, Six, and Seven) are complimented by one laboratory based experiment (Chapter Five).

In Chapter Four, 20 lysimeters were used to quantify the amount of N_2O emitted from winter dairy forage soil, and to test the effectiveness of DCD, biochar, and their combination in mitigating N_2O emissions. To simulate a forage crop, kale was grown in the lysimeters, before being harvested and the soil trampled using a mechanical method. Fresh dairy cow urine was applied to the trampled soil at a rate equivalent to 700 kg N ha^{-1} to simulate animal urine patches, while inter-urine patches received no urine.

In the laboratory based experiment of Chapter Five, the effect of animal trampling was investigated, as was the effect of dairy cow urine, and DCD on N_2O emissions, soil mineral N concentrations, and the abundance of microbial denitrifying genes within the soil.

Chapter Five was run concurrently with Chapter Six – a field-based experiment using 32 lysimeters and companion soil plots. Chapter Six studied the same factors as Chapter Five, and included measurements of the moisture, bulk density, and air permeability of the soil. In Chapter Six, urine was applied at a lower N-rate than in Chapter Four, equivalent to 400 kg N ha^{-1}, in response to new research showing cows fed kale had lower urine-N concentrations than cows grazing pasture.

The final experimental component, Chapter Seven, refined the work in Chapter Six, and included an extra treatment replicate to reduce treatment variability. Urine enriched with ^{15}N-labelled urea was applied to the soil to help trace the fate of the urine.

Full details of the methodology used in each experiment are contained within the individual experimental chapters.

Chapter Four – The effectiveness of the nitrification inhibitor, dicyandiamide, in combination with biochar, to reduce nitrous oxide emissions from a simulated winter grazed forage crop soil – a field experiment

4.1 Introduction

The agricultural sector produced about half of New Zealand's (NZ's) greenhouse gas (GHG) emissions in 2012, and about 30% of those emissions were nitrous oxide (N_2O) (Ministry for the Environment 2014). Nitrous oxide (N_2O) is a powerful GHG, with a global warming potential about 300 times that of carbon dioxide (CO_2) (IPCC 2013). Direct and indirect emissions from animal excreta deposited onto soil are responsible for the majority of NZ's N_2O emissions. The amount of N_2O emitted in NZ increased by 32% between 1990 and 2012, primarily as a result of an increase in the number of dairy cows (Ministry for the Environment 2014). An increasing number of dairy herds graze high yielding forage crops such as brassica during the winter (Drewry and Paton 2005), which can lead to soil compaction and a large number of urine patches in a small area due to the high stock density (Figure 4.1). Both urine deposition and animal trampling favour soil denitrification and contribute to elevated N_2O emissions (Menneer *et al.* 2005, van Groenigen *et al.* 2005b, Ball *et al.* 2012).

Figure 4.1 Dairy cows strip-grazing forage brassica at Lincoln University's Ashley Dene research farm.

Nitrification inhibitors such as dicyandiamide (DCD), have been shown to reduce agricultural N_2O emissions from grazed pasture (Di and Cameron 2002, Di *et al.* 2010, de Klein *et al.* 2011). DCD has also been shown to reduce N_2O emissions when applied to compacted soil (Ball *et al.* 2012), and to grazed winter feedlots (Smith *et al.* 2008b). DCD inhibits the growth and activity of soil ammonia oxidising bacteria, thus slowing down the nitrification process. Therefore there is a reduction in the amount of N_2O emitted from nitrification. There is also a reduction in the amount of nitrate (NO_3^-) available for denitrification. Furthermore, DCD also reduces nitrate leaching (Di and Cameron 2002, 2004a), which reduces indirect N_2O emissions (Clough *et al.* 2007).

Biochar is the carbon rich remains of organic matter heated in the absence of oxygen (Lehmann and Joseph 2009). Biochar not only has the potential to sequester atmospheric CO_2 in soil (Clough and Condron 2010, Sohi *et al.* 2010), but has also been used as an agricultural soil amendment to mitigate N_2O emissions (Zhang *et al.* 2010, Taghizadeh-Toosi *et al.* 2011). The mechanisms by which biochar supresses N_2O emissions is the subject of considerable ongoing research. Biochar is thought to produce physical, chemical, and biological changes within soil, leading to various alterations of the nitrogen cycle (Lehmann and Joseph 2009). Several authors (Ding *et al.* 2010, Hale *et al.* 2013, Hollister *et al.* 2013) have demonstrated the ability of biochar to remove ammonium (NH_4^+) from solution, while Sarkhot et al. (2013) found biochar removed up to 18% of NH_4^+ from dairy effluent.

Biochar could have the potential to mitigate N_2O emissions from urine amended soil (Singh *et al.* 2010, Taghizadeh-Toosi *et al.* 2011), however, to date, it's potential to reduce N_2O emissions in winter forage grazing systems, either alone, or in combination with DCD, has not been quantified. The purpose of this research was to study the capacity of DCD and biochar, alone, or together, to reduce N_2O emissions from a dairy winter forage grazing system. It was hypothesised that the inhibition of the nitrification process combined with the enhanced capacity of cation retention provided by biochar would provide a synergistic effect on the mitigation efficacy of DCD and biochar.

4.2 Methods

4.2.1 Soil and climate

Soil and lysimeter collection

A Balmoral stony silt loam, classified as Pallic Orthic Brown Soil (Hewitt 2010); Udic Haplustept loamy skeletal (Soil Survey Staff 2010), typical of soils used for dairy winter forage grazing in the Canterbury region, South Island of New Zealand, was used in this study. Developed from gravelly glacial outwash alluvium and loess, the Balmoral soil consists of a thin, sandy silt loam top soil containing about 30% stones. Below the top soil, the stone and sand content increases substantially, making this soil very free draining (Figure 4.2).

Figure 4.2: **A stony Balmoral soil at the site where the lysimeters were extracted. Note camera case for scale.**

Twenty lysimeters were collected from a Balmoral soil at Lincoln University's Ashley Dene farm (43°38'55"S, 172°20'39"E), during December 2010, using the method described by Cameron et al. (1992). To create the undisturbed soil monoliths, steel casings (50 cm diameter by 70 cm depth) were pushed into the soil in small increments, while the surrounding soil was gently scraped away, leaving

an undisturbed soil column within the casing. Any stones that impeded the process were removed from the soil. The edges of the soil column were sealed to the casing with molten petroleum jelly, to prevent edge flow effects, before a cutting plate was inserted beneath the base of the lysimeter housing (Figure 4.3). The lysimeters were then installed in a lysimeter facility at Lincoln University (43°39'04"S, 172°28'03"E), level with the surrounding soil. The outside of the monoliths were back-filled to maintain the climatic conditions of the surrounding field (Figure 4.4, Figure 5.5).

The pasture in the lysimeters was sprayed with herbicide during December 2010, before being planted with the fodder crop kale (*Brassica oleracaea* cv. Regal). After emergence, the kale was thinned to a density of 20 plants m^2, and allowed to reach maturity. The mature kale was harvested on 21st June 2011 to simulate grazing, prior to applying the treatments to the lysimeters.

Figure 4.3: **Inserting the bottom plate of the lysimeter using a hydraulic ram. The bottom plate is secured to the lysimeter using the steel rods visible at the top of the picture.**

Climate

The average annual rainfall in the Canterbury region is about 650 mm, and the average annual temperature about 12.1 °C. Water input was maintained at the 75th percentile of the regional average using simulated rainfall when required. A full description of the irrigation scheduling can be found in Malcolm (2013). Rainfall at the experimental site was measured by a tipping bucket rain gauge (model

OSK 15180-T, Ogawa Seiki, Japan), while soil and air temperature was measured by Campbell Scientific CS107 sensors at the same facility.

4.2.2 Treatments

Four treatments were randomly allocated to the lysimeters on 29th June 2011: urine (U); urine plus DCD (UD); urine plus biochar (UB); and urine plus DCD and biochar (UDB). A control (C) was used to provide background N_2O emission data. All treatments, including the control, were replicated four times. Following harvest of the kale, the soil was wetted to field capacity by adding 10 mm of simulated rainfall. The UB and UDB treatment lysimeters were amended with biochar (Pacific Pyrolysis, NSW, Australia) (Table 4.1) at the rate of 5 t ha^{-1}, which was incorporated into the top 5 cm of the soil (Figure 4.4). The soil in all lysimeters was then trampled using cow hoof simulation equipment designed to provide approximately 200 kPa – similar to the pressure exerted by an adult cow hoof (Di *et al.* 2001). The surface of the soil was completely trampled twice, to simulate the heavy grazing typical for winter forage. DCD was dissolved in water and applied to the UD and UDB treatment lysimeters at a rate equivalent to 20 kg ha^{-1} using hand held sprayers. Fresh urine was collected from non-lactating dairy cows that were feeding on kale, and analysed for total N concentration. The urine was standardised to 7.0 g N L^{-1} by adding urea and glycine, and applied to all lysimeters, except the controls, at a rate equivalent to 700 kg N ha^{-1}. The controls received an equivalent volume of water.

Table 4.1: Selected properties of the biochar used during the field experiment, 2011,

Property	Measurement
Feedstock	Radiata pine chips
Pyrolysis temperature (°C)	550
Bulk Density (g cm^{-3})	0.25
pH	8.55
Total Carbon (g kg^{-1})	830
Total Nitrogen (g kg^{-1})	3.1
C:N ratio	269:1
Nitrate (mg N kg^{-1})	0.11
Ammonium (mg N kg^{-1})	5.33

Figure 4.4: **Soil in the lysimeters prior to trampling. Note how the addition of biochar to the lysimeter in the foreground produced a darker colour to the soil relative to the lysimeter in the background which did not have biochar added. Also note the aluminium rings sealed to the lysimeter casings to provide a water trough for N_2O sampling.**

4.2.3 Nitrous oxide emissions

A closed chamber method, similar to that of Hutchison & Mosier (1981), was used to determine N_2O emissions. A water filled trough on top of each lysimeter casing provided a gas tight seal for the metal and polystyrene chamber used during sampling (Figure 4.4, Figure 4.5). At each sampling time, chambers were placed on top of the lysimeters for a total of 40 minutes, with three samples taken 20 minutes apart, i.e. t=0, t=20, and t=40 minutes. Samples were taken through a rubber septum located in the top of each chamber using a 60 ml syringe and hypodermic needle, and placed in 6 ml glass vials. Samples for N_2O analysis were taken between 12:00 and 14:00 hours at each sampling event. Nitrous oxide concentration was analysed using gas chromatograph (SRI 8610GC, SRI Instrument, C.A., USA).

This study focused on the winter grazing period, and consequently N_2O measurements were taken for four months, from June to October 2011, representing the approximate time a farmer may leave the paddock fallow before re-sowing with another crop.

Figure 4.5: **Lysimeters during N_2O sampling at the lysimeter facility. The grey metal and polystyrene chambers were sealed to the lysimeters in a water trough.**

4.2.4 Data analysis

Nitrous oxide emission rates were calculated from the increase in concentration of N_2O between the first and second, and second and third gas samples taken at each sampling event (Hutchison & Mosier, 1981). Daily N_2O fluxes were then calculated on the assumption that the calculated hourly flux represented the average hourly flux for that day. Total N_2O emissions were calculated by integrating the daily emission fluxes. Statistical analysis of the data was performed using the GenStat (Version 16) software package.

The emission factor (EF_3), or proportion of applied N that was emitted as N_2O was calculated using Equation 4.1 (de Klein *et al.* 2003):

$$EF(\%) = \frac{N_2O\text{-}N\ total\ (treatment) - N_2O\text{-}N\ total\ (control)}{UrineN\ (applied)}\ x\ 100 \qquad (4.1)$$

where *EF(%)* is the emission factor, *N_2O-N total (treatment)* is the cumulative total N_2O emitted from a urine treatment, *N_2O-N total (control)* is the cumulative total N_2O emitted from the comparative no-urine treatment, and *UrineN (applied)* is the amount of N added as urine.

4.3 Results

4.3.1 Climate

During the experimental period, 342 mm of rain fell, which was supplemented with 122 mm irrigation to match the 75th percentile of rainfall for the region (Figure 4.6). Average daily air temperature ranged from 0.6˚C to 17.6 ˚C (Figure 4.7).

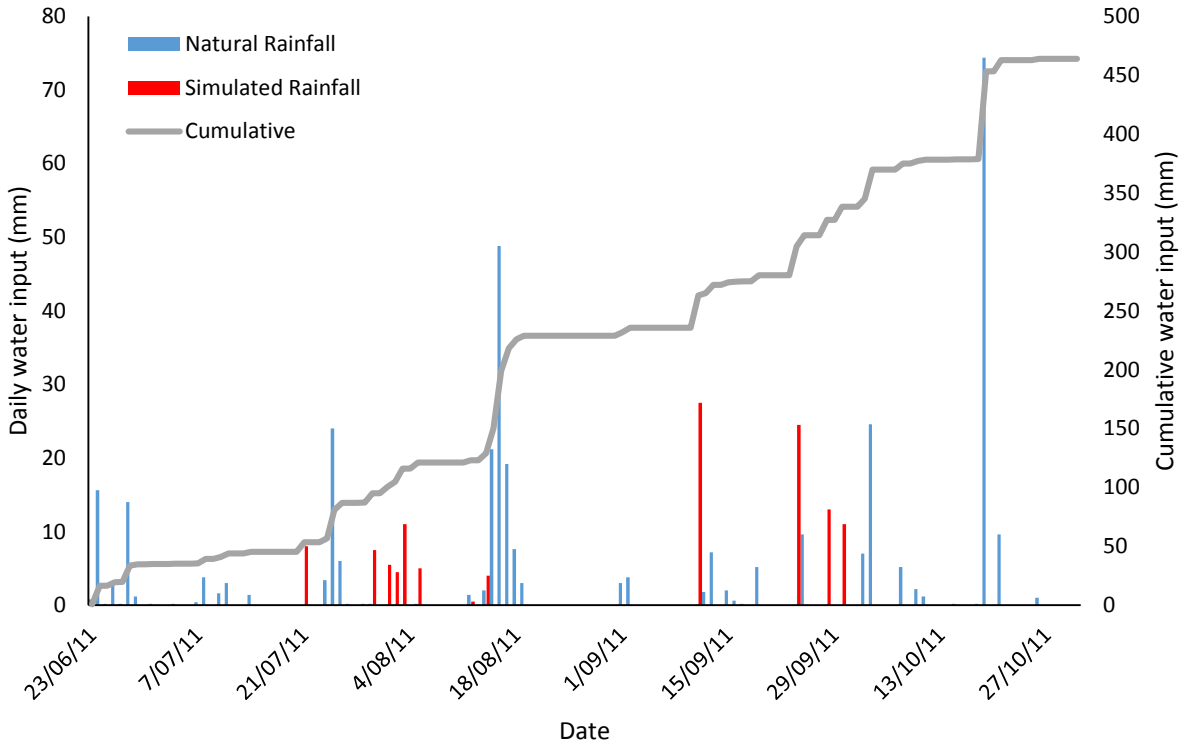

Figure 4.6: **Total water input to the lysimeters from 23rd June to 30th October 2011. Cumulative line includes both natural and simulated rainfall.**

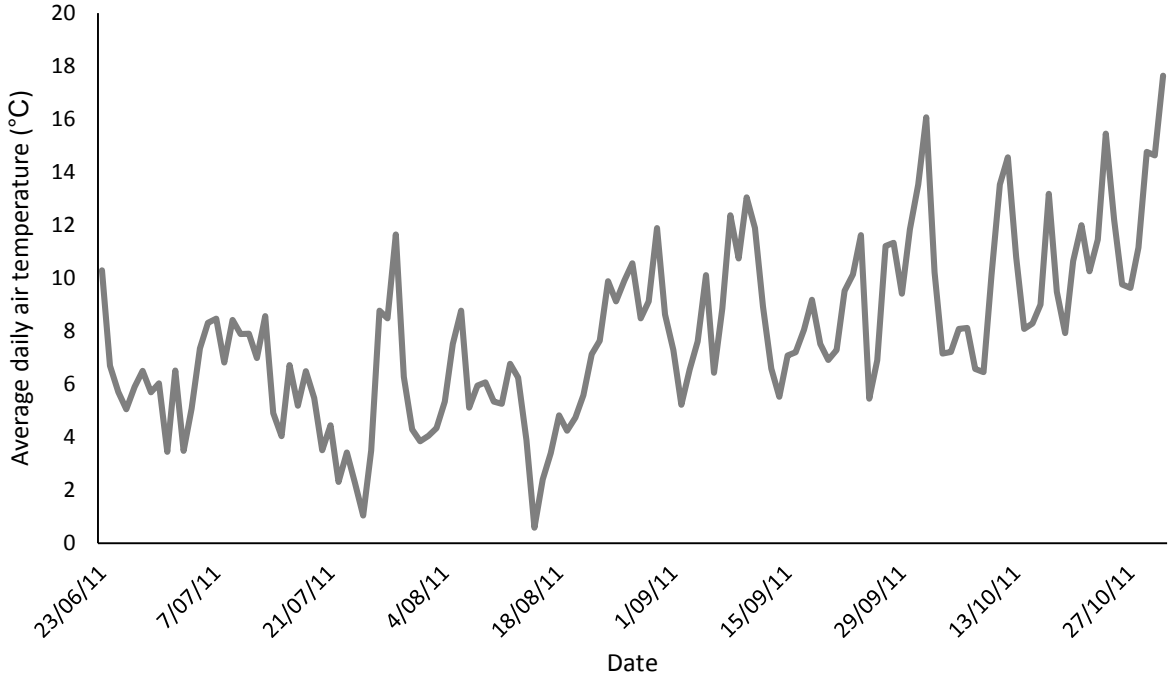

Figure 4.7: Average daily air temperature at the lysimeter facility from 23rd June to 30th October 2011.

4.3.2 Nitrous oxide emissions

Daily N_2O emissions

Dairy cow urine applied at 700 kg N ha^{-1} produced peak N_2O emissions of 257 g N_2O-N ha^{-1} day^{-1} in the U only treatment, compared with 33 g N_2O-N ha^{-1} day^{-1} in the control (Figure 4.8). Applying DCD to the soil reduced peak N_2O emissions by 58% to 108 g N_2O-N ha^{-1} day^{-1} in the UD treatment. Incorporating biochar into the soil produced a peak emission of 295 g N_2O-N ha^{-1} day^{-1} from the UB treatment. The peak N_2O emission from the UDB treatment of 146 g N_2O-N ha^{-1} day^{-1} was not significantly different from the UD treatment. The peak N_2O emissions for all urine amended treatments were recorded on 12th September 2011 (Figure 4.8).

Total N_2O emissions

The addition of dairy cow urine increased total N_2O emissions from the trampled stony Balmoral soil from 0.5 kg N_2O-N ha^{-1} to 13.6 kg N_2O-N ha^{-1} (Table 4.2). The application of DCD reduced total N_2O emissions by 68% to 4.3 kg N_2O-N ha^{-1} (Table 4.2). Biochar did not significantly decrease N_2O emissions, with N_2O emissions from the UB treatment reaching 12.2 kg N_2O-N ha^{-1}, and the UDB treatment emitted a total of 5.5 kg N_2O-N ha^{-1}.

Table 4.2: **Total cumulative N_2O emissions, reduction in N_2O emissions due to mitigation strategy, and proportion of applied N emitted as N_2O from lysimeters from 26th June to 30th October 2011.**

Treatment	Total emissions (kg N_2O-N ha^{-1})	% Reduction	Emission factor (% of applied N)
C	0.5	-	-
U	13.6	-	1.9
UB	12.2	10	1.7
UD	4.3	68	0.5
UDB	5.5	60	0.7
LSD# (0.05)	6.7	-	-

#Least significant difference ($P < 0.05$), controls omitted from LSD calculation due to unequal variance between treatments.

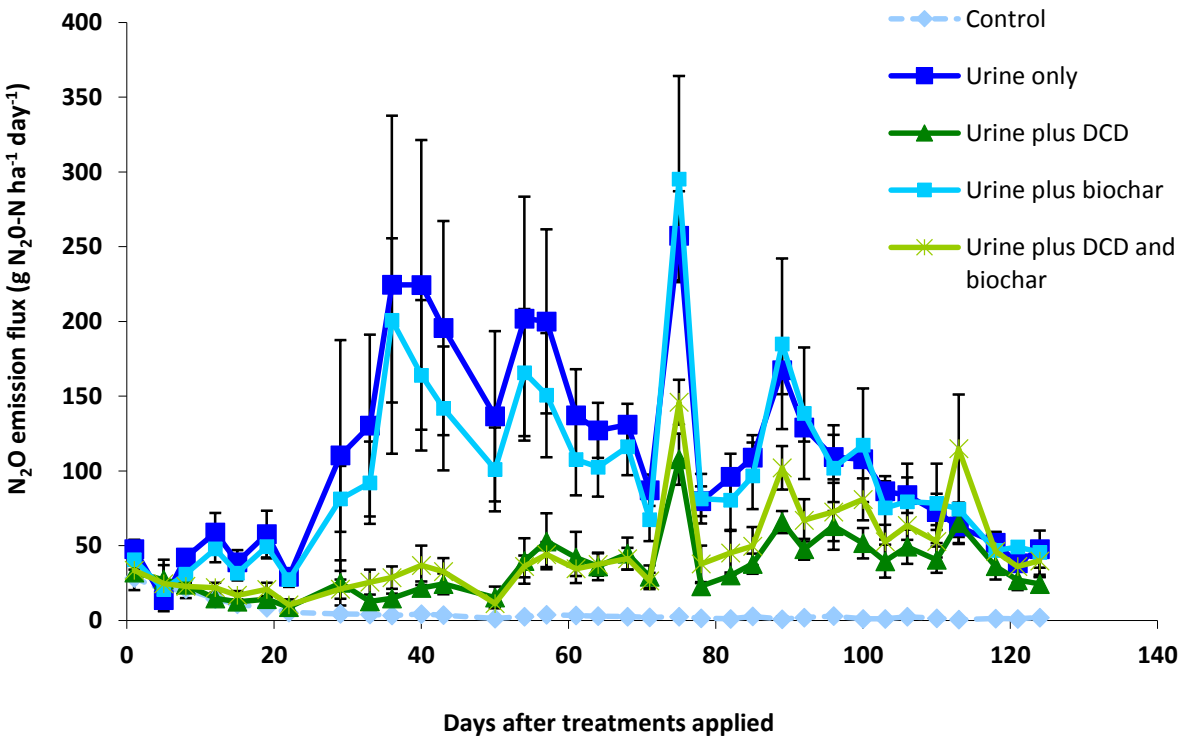

Figure 4.8: **Nitrous oxide emission flux from lysimeters from 26th June to 30th October 2011. Error bars represent one standard error of the mean.**

4.4 Discussion

Results from this study show that treating winter grazed dairy forage soil with the nitrification inhibitor, DCD, was effective in reducing N_2O emissions. DCD reduced total N_2O emissions by 68%, (Table 4.2) and reduced peak N_2O emissions (day 75) by 58% in urine affected areas of the soil (Figure 4.8). The reduction in N_2O emissions from the application of DCD presented in this study is similar to

those reported elsewhere, in both pastoral and forage soil (Di *et al.* 2007, Smith *et al.* 2008b, Di *et al.* 2010).

In contrast to DCD, the incorporation of biochar into the Balmoral soil had no significant effect on N_2O emissions from dairy cow urine areas. There was also a lack of a synergistic effect between DCD and biochar on N_2O emissions. However, other authors have reported that biochar can reduce N_2O emissions. For example, in fertilized rice paddy field soil, Zhang et al. (2010) reported a decrease in N_2O emissions of 21-28% when biochar was added at rates of 10 – 40 t ha^{-1}, and Wang et al. (2011) found biochar reduced N_2O emission from rice paddy soil by 61% when applied at 50 t ha^{-1}. Similarly, when applied at a rate equivalent to 15 t ha^{-1}, biochar reduced N_2O emissions from soil amended with a N-rich slurry by 47% (Bruun *et al.* 2011). Importantly, Bruun et al. (2011) found no significant reduction in N_2O emissions when biochar was applied at a lower rate equivalent to 5 t ha^{-1} (similar to this study), suggesting that N_2O emission reductions from biochar may be rate dependant. In this study biochar was applied at 5 t ha^{-1} because this was considered a practical amount for farmers to apply and work into the soil using conventional methods.

Results reported in the literature vary, as some authors have been unable to detect N_2O emission reductions when biochar was applied at rates higher than 5 t ha^{-1}. For example, at 10 t ha^{-1}, biochar did not significantly alter N_2O emissions from subtropical pasture (Scheer *et al.* 2011), and Clough et al. (2010) found no change in N_2O emission from urine amended soil when biochar was added at a rate of 30 t ha^{-1}. However, Singh et al. (2010) found that biochar at 10 t ha^{-1} was effective in reducing N_2O emissions from soil.

Some authors (e.g. Clough *et al.* 2010, Singh *et al.* 2010, Wang *et al.* 2011) have shown a temporal change in N_2O emissions, such as a delay in peak emissions when biochar was added relative to control treatments. However, N_2O emissions from the biochar treatments in this study were not significantly different to the other urine treatments, indicating that the biochar did not have a significant effect on emissions.

The emission factors – or proportion of applied N released by the soil as N_2O – reported in this study were within the range of values presented in the review by Cameron et al. (2013), including a similar study on a stony soil in Canterbury (Di and Cameron 2005). Furthermore, the 68% reduction in N_2O emissions due to DCD in a forage system, as presented here, is very similar to the rate of 67% adopted for pastoral grazing included in reporting to the IPCC by the New Zealand Government (Ministry for the Environment 2013). The DCD reduction rate presented here is also comparable to the average reduction rate of 54% calculated in a review of published results by de Klein et al. (2011).

On 12th September 2011, a spike in N_2O emission fluxes was recorded for all urine amended treatments, which was inconsistent with the declining trend in emission fluxes recorded prior to, and

following, 12th September (Figure 4.8). Changes in N substrate, soil or air temperature, or soil moisture status (De Klein and Van Logtestijn 1996, Cameron *et al.* 2013) could be potential causes of the anomalous emission flux. As no N had been added to the lysimeters between sampling events, it was unlikely that the N substrate had changed considerably from the previous sampling. Average air temperature on 12th September was about 9°C, and the air temperature at the time of sampling was about 17°C, which was not dissimilar to the air temperature at other sampling events in September. A similar situation was observed by Ball *et al.* (2012), who noted a substantial spike in N_2O emissions following 43 mm of rainfall. On the day prior to the anomalous emission peak in this study, 27.5 mm of simulated rainfall was applied to the lysimeters which followed about three weeks with almost no rainfall (Figure 4.6).

The simulated rainfall on 11th September would have saturated the soil, and possibly stimulated denitrification under the anaerobic conditions present within the trampled soil. A period of nitrification may have occurred during the warming conditions in three weeks prior to 12th September, which may have provided the substrate for denitrification to occur. However, similar spikes in the N_2O emission flux were not recorded after other rainfall events, such as those that occurred on 16th August, and 19th October 2011. Furthermore, no information was available on the mineral nitrogen concentration in the soil, the nitrifying or denitrifying microbial population, or on the soil moisture status at the time of sampling, all of which could help provide a possible explanation for the emission pattern observed. These measurements were therefore added to subsequent studies reported in this thesis.

4.5 Conclusions

The application of a nitrification inhibitor, DCD, reduced N_2O emissions from urine affected areas of winter grazed dairy forage soil by 68%, while incorporating biochar into the soil had no significant effect on emissions. Combining biochar with DCD did not provide improvement in the efficacy of DCD to reduce N_2O emissions. The rate of biochar addition chosen in this study was at the lower end of application rates published by other authors. However, no practical method exists to apply biochar to agricultural fields at rates as high as 30 t ha^{-1}. As there was no statistically significant difference between the treatments with biochar, and the comparable treatments without biochar, and given that higher rates of biochar application were unlikely to be employed by farmers, biochar was not used in subsequent studies in this thesis.

Chapter Five – The effect of soil moisture, soil compaction, and the nitrification inhibitor, dicyandiamide, on nitrous oxide emissions from dairy cow urine affected soil – a laboratory experiment

5.1 Introduction

Nitrous oxide (N_2O) is a powerful greenhouse gas (GHG), with a long atmospheric residence time of about 120 years (IPCC 2013). The agricultural sector produced about half of New Zealand's (NZ's) GHG emissions in 2012, and 30% of those emissions were nitrous oxide (N_2O) (Ministry for the Environment 2014). Direct and indirect emissions from animal excreta deposited onto soil are responsible for the majority of NZ's N_2O emissions.

The amount of N_2O emitted in NZ increased by 32% between 1990 and 2011, largely as a result of an increase in the number of dairy cows (Ministry for the Environment 2014). In agricultural soil, N_2O is predominantly produced during the microbial processes of nitrification and denitrification (Firestone and Davidson 1989, Groffman *et al.* 2006). Soil can be subject to animal trampling damage and concentrated urine deposition when dairy herds are taken to graze high yielding forage crops during the winter (Drewry and Paton 2005) (Figure 5.1). Both urine deposition and animal trampling favour denitrification in soil and contribute to elevated N_2O emissions (Menneer *et al.* 2005, van Groenigen *et al.* 2005b, Ball *et al.* 2012). However, information on the amount of N_2O produced by dairy cows grazing forage crops in winter, or ways to mitigate those emissions, is insufficient (Ministry for the Environment 2011).

Nitrification inhibitors such as dicyandiamide (DCD), have been used successfully to mitigate agricultural N_2O emissions from grazed pasture (Di and Cameron 2002, Di *et al.* 2010, de Klein *et al.* 2011). DCD has also been effective in reducing N_2O emissions when applied to compacted soil (Ball *et al.* 2012), and to grazed winter feedlots (Smith *et al.* 2008b). In Chapter Four of this thesis, the application of DCD to compacted soil following urine deposition was reported to have reduced total N_2O emissions by 68%, however this was affected by climatic conditions.

Measuring the abundance of denitrifying gene copies within soil has been used to estimate denitrification potential (e.g. Throback *et al.* 2004, Henry *et al.* 2006, Dandie *et al.* 2007), and while soil moisture has been positively correlated with N_2O emission (e.g. Davidson 1993, van Groenigen *et al.* 2005a), few have measured the abundance of denitrifying gene copies in relation to soil moisture.

Recently, Di *et al.* (2014) found that the higher the volumetric soil moisture content, the higher the abundance of denitrifying gene copies and N_2O emissions from an agricultural soil. Although Di *et al.* (2014) calculated the volumetric soil moisture content, others have used the soil water filled pore space (WFPS) as a measure of soil moisture in relation to denitrification and N_2O emissions (e.g. Weier *et al.* 1993, Dobbie *et al.* 1999, Ruser *et al.* 2006).

The aim of the present laboratory experiment was to study the influence of volumetric soil moisture content and WFPS on N_2O emissions, the mineral N concentration, and the abundance of denitrifying gene copies present in the soil, without the influence of fluctuating temperature and moisture that was experienced in the field (i.e. Chapter Four). Given the success of DCD in reducing N_2O emissions in the previous field experiment, DCD was incorporated into the current experiment to evaluate its effect on nitrification rate and N_2O emissions.

Figure 5.1: **Heavy trampling of soil by dairy cows grazing on a forage crop during winter at Lincoln University's Ashley Dene Research Farm, 8th June 2012.**

5.2 Methods

5.2.1 Soil

A Balmoral stony silt loam, classified as Pallic Orthic Brown Soil (Hewitt 2010); Udic Haplustept loamy skeletal (Soil Survey Staff 2010), typical of soils used for dairy winter forage grazing in the Canterbury region, South Island of New Zealand, was used in this study. Developed from gravely glacial outwash alluvium and loess, the Balmoral soil consists of a thin, sandy silt loam top soil containing about 30% stones.

Fifty litres of Balmoral soil was collected from Lincoln University's Ashley Dene farm (43°38'55"S, 172°20'39"E). Before collecting the soil, the vegetation was removed, and the top 10 cm of soil was sieved using a 5 mm mesh sieve to remove stones. The moisture content of the bulk soil was determined gravimetrically by drying 5 replicates of representative samples at 105°C for a minimum of 24 hours. The soil was stored at 4°C, until use.

5.2.2 Treatments

Ten treatments, each with four replicates, were established to test the hypothesis that denitrification would be enhanced in urine-affected, moist, compacted soil (Table 5.1). The effects of different soil moisture levels expressed as water filled pore space (WFPS) and as volumetric water content (θ_v), two soil bulk densities, the effect of dairy cow urine, and the effect of DCD on N_2O emissions, mineral N content, and denitrifying microbial gene abundance were determined.

A closed chamber method based on Hutchinson and Mosier (1981) was used to determine nitrous oxide (N_2O) emissions. Four hundred grams of oven dry equivalent soil was placed into 40 glass jars, 575 ml in capacity. To determine soil mineral N, and for microbial assays, 480 centrifuge tubes, 50 ml in capacity, were filled with 40 g of oven dry equivalent soil. Before adding the soil, the mass of the centrifuge tubes and glass jars were determined. The centrifuge tubes were found to be of a sufficiently uniform mass, and the glass jars were standardised by adding mass to the outside of the jar until they weighed within 0.1 g of each other. The soil in both the glass jars and the centrifuge tubes was compacted to the required density of 1.3 g dry soil cm^{-3} for the trampled treatments, and 1.0 g dry soil cm^{-3} for the non-trampled treatments. To achieve a consistent density of 1.3 g dry soil cm^{-3} throughout the soil column, the soil of the trampled treatments was compacted in four layers (Figure 5.2).

Table 5.1: Soil properties and details of treatments.

Treatment name[#]	Bulk density (g cm^{-3})	Urine-N (mg N g $soil^{-1}$)	DCD (mg DCD g $soil^{-1}$)	Water filled pore space (%)	θ_v (v/v%)
T25	1.3	0	0	50	25
TU25	1.3	583	0	50	25
TUD25	1.3	583	16.7	50	25
TD25	1.3	0	16.7	50	25
T41	1.3	0	0	80	41
TU41	1.3	583	0	80	41
TUD41	1.3	583	16.7	80	41
NT50	1.0	0	0	80	50
NTU50	1.0	583	0	80	50
NTUD50	1.0	583	16.7	80	50

[#] 'T' indicates simulated trampled treatments, 'NT' indicates no simulated trampling, 'U' indicates urine was applied, 'D' indicates DCD was applied, while '*25'*, '*41'* or *'50*' indicates θ_v.

Water filled pore space (WFPS) was calculated using Equation 5.1 (Linn and Doran 1984):

$$WFPS = \frac{\theta_v}{TP} \quad (5.1)$$

where θ_v is the percent volumetric water content (%θ_m)(P_B), and TP is the percent total soil porosity (1 - P_B/P_P) (100), where P_P is the soil particle density (assumed to be 2.65 Mg/m^3), P_B is the soil bulk density (Mg/m^3), and θ_m is the gravimetric water content (g/g).

De-ionised water was added to the soil-filled jars and tubes to bring the soil to the required water content, with allowances made for the addition of urine to the appropriate treatments. Metal lids with two holes approximately 1 cm in diameter were screwed to the jars, and parafilm was stretched over the top of each centrifuge tube. A small hole was made in the parafilm to allow gaseous exchange between the headspace of the incubation vessel and the atmosphere. The soil was left for two days to allow the soil moisture to equilibrate, before the mass of each tube and jar was checked and adjusted with de-ionised water if required.

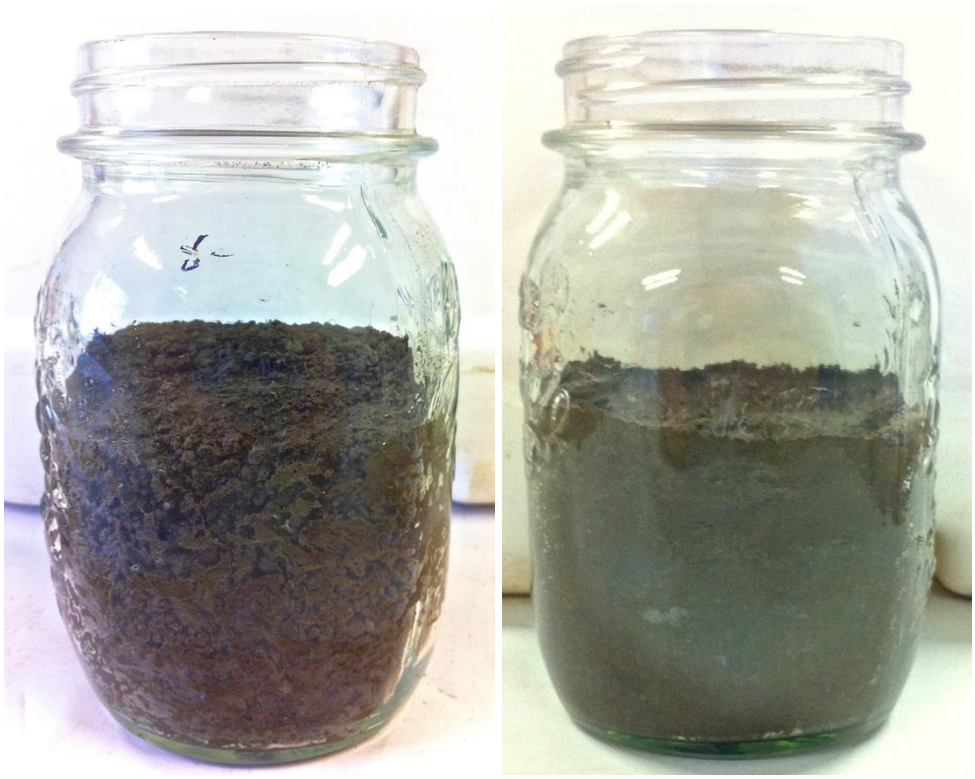

Figure 5.2: **Incubation vessels containing soil at two bulk densities, both at 80% water filled pore space. Note pore spaces between aggregates in the not-trampled soil (1.0 g cm^{-3}, left) that are not apparent in the tramped soil (1.3 g cm^{-3}, right).**

Each glass jar was randomly allocated a number from 1 to 40 and placed in an incubator set to maintain 10°C. The centrifuge tubes were grouped in batches of 40 tubes (four replicates of each of the ten treatments) and arranged in racks according to the same random numbering system derived for the glass jars. The racks were placed in the same incubator as the jars (Figure 5.3).

Urine was collected from lactating dairy cows and analysed for N concentration using an Elemental Analyser (Elementar Vario MAX CN), before being standardised to 7 g N L^{-1} using de-ionised water. The standardised urine was applied to the top of the soil in the tubes and jars of the NTU25, TU41, and TU25 treatments at a rate of 583 mg N kg dry $soil^{-1}$. The soil-N concentration chosen represents the average N concentration in the soil of a cow urine patch with an N loading of 700 kg N ha^{-1}, assuming a soil density of 1.2 g cm^{-3}, and a urine infiltration depth of 10 cm. DCD was dissolved in a separate aliquot of cow urine to achieve a concentration of 0.20 g DCD L^{-1}, which was equivalent to 16.7 mg DCD kg dry $soil^{-1}$, or 20 kg DCD ha^{-1}. The DCD and urine solution was added to top of the soil in the tubes and jars of the NTUD25, TUD41, and TUD25 treatments. DCD was also dissolved in de-ionised water and applied to the top of the soil in the tubes and jars of the TD25 treatments to achieve a concentration of 16.7 mg DCD kg dry $soil^{-1}$. All tubes and jars were re-weighed, and if necessary, brought back to the target mass using de-ionised water.

The samples were kept as far from the fans in the incubator as possible, and throughout the duration of the experiment, a full tray of water was kept near the top of the incubator to limit the rate of evaporation from the samples. The moisture of the soil in each of the tubes and jars was maintained by weighing weekly, and adjusted to the target mass with de-ionised water if necessary.

Figure 5.3: **Soil-filled jars for N_2O emission calculations near the top of the incubator, centrifuge tubes for microbial and mineral N analysis in the lower part of the incubator.**

5.2.3 Nitrous oxide emissions

Nitrous oxide emissions from the incubated soil were calculated from gaseous samples taken from the headspace of each of the 40 soil-filled jars. Two samples were taken 30 minutes apart, as preliminary work showed a linear increase in N_2O emission over the 30 minute time period. At each sampling time, the jars were taken from the incubator, the lids removed and replaced with sealed metal lids with a rubber septum. Immediately upon placing the lid on each jar, a 12 ml sample of headspace gas was taken through the rubber septum using a 60 ml syringe and hypodermic needle, and placed in a 6 ml glass vial (Figure 5.4). The jars were left for 30 minutes to allow N_2O to accumulate in the headspace of the jar, before a second sample was taken through the rubber septum and placed into another glass vial. After gaseous samples were taken, the lids with the septum were removed and replaced with the lid with holes. The moisture of the soil was maintained by weighing each jar and bringing the mass to the pre-determined target using de-ionised water if necessary. The rate of N_2O

emission was calculated by comparing the concentration of N_2O in the headspace between the two samples. Samples were taken twice weekly for the first three months, and then approximately weekly for the remainder of the experiment. Gaseous samples were taken for 196 days, at which time N_2O emissions from the urine-amended soil had returned to background levels.

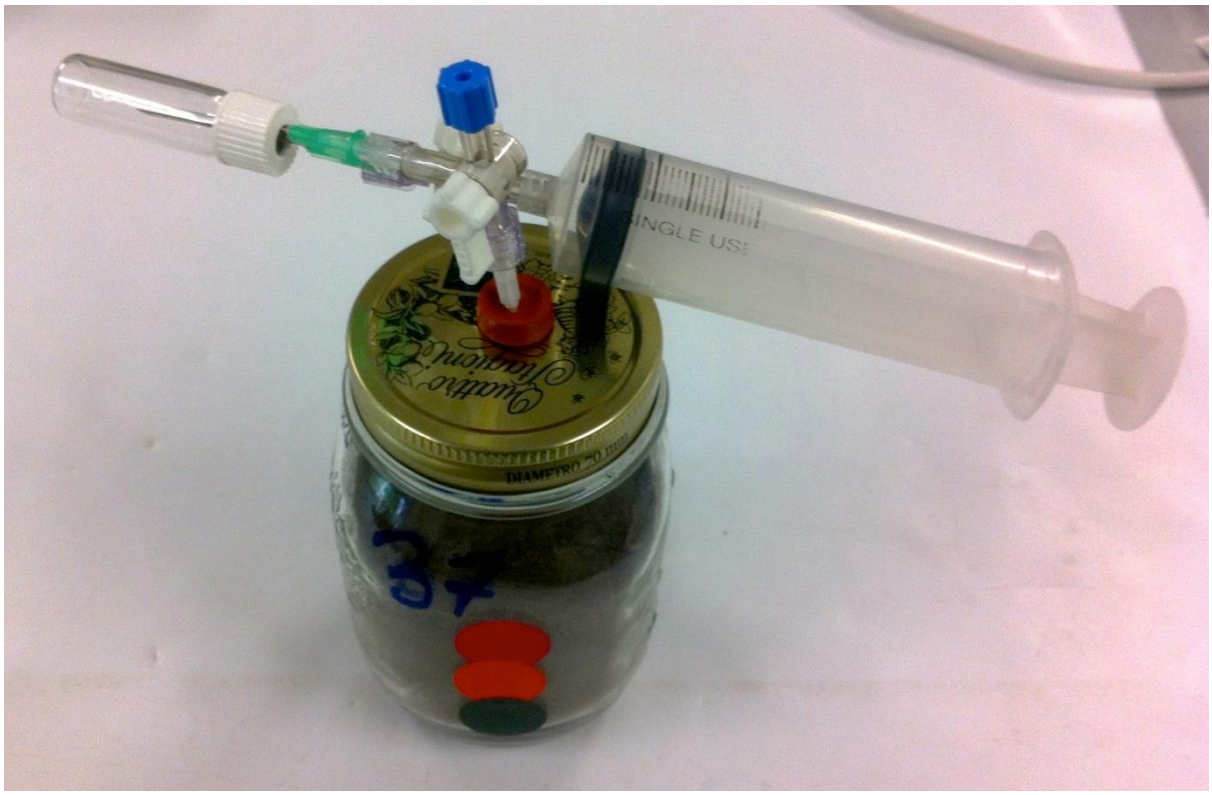

Figure 5.4: **Samples were taken for N_2O analysis by placing a sealed lid on each of the jars, extracting headspace gases through a rubber septum using a 60 ml syringe, and transferring the gases to a glass vial. The tap placed at the top of the jar allowed the headspace to remain sealed, while allowing for periodic opening to take samples.**

5.2.4 Soil mineral nitrogen

The soil contained within the centrifuge tubes was used to determine mineral N concentration on eleven occasions throughout the duration of the experiment. At each sampling event, one batch of 40 tubes (four replicates of ten treatments) was removed from the incubator. The contents of the tubes were emptied into individual plastic bags, and mixed thoroughly. For each of the 40 samples, five grams of mixed soil was placed in another centrifuge tube before adding 25 ml of 2 M KCl. The tubes were then placed in a rack and shaken for 1 hour. After shaking, the solution was centrifuged for 10 minutes, before being filtered through Whatman #42 (or equivalent) filter paper. The filtrate was frozen until being analysed using a flow injection analyser (Tecator Inc. Sweden) for NH_4^+ and NO_3^- concentration.

The soil moisture content was standardised during the initiation of the experiment. However, to confirm soil moisture at the time of sampling, on four occasions, soil from one replicate of each treatment was dried in an oven at 105°C for a minimum of 24 hours. Regular monitoring of the tube and soil mass was sufficient to preserve the soil moisture to within 2% of the targeted soil moisture.

To confirm that the centrifuge tubes provided a sufficiently similar storage environment to the glass jars, at the completion of the experiment, a column of soil was taken from each of the glass jars and analysed for NH_4^+ and NO_3^- concentration (as described above). The concentration of NH_4^+ and NO_3^- in the soil stored in glass jars was compared to that of the soil stored in centrifuge tubes.

5.2.5 Soil DCD concentration

In combination with each sampling event outlined in 5.2.4, five grams of soil from each of the TUD25, TD25, TUD41, and NTUD50 treatments were placed in centrifuge tubes, before adding 25 ml of de-ionised water. The tubes were then placed in a rack and shaken for 1 hour. After shaking, the solution was centrifuged for 20 minutes, before being filtered through Whatman #42 (or equivalent) filter paper. The filtrate was frozen before being analysed using a High Performance Liquid Chromatograph (Shimadzu, Japan) for DCD concentration.

5.2.6 Microbial gene abundance

Additional samples of soil for assessing selected microbial gene abundance were taken at the same times as the samples were taken for mineral N and DCD assays. The abundance of the NH_4^+ oxidising *amoA* gene, the NO_2^- reductase gene *nirS*, and the N_2O reductase gene *nosZ*, were analysed from soil samples taken from the three urine-only treatments (NTU50, TU41, and TU25). Approximately five grams of soil was packed into 6 ml plastic vials and stored at -80°C until analysis, using the methods described by Di *et al.* (2014) (Table 5.2). Briefly, DNA was extracted from the soil using MoBio Powersoil™ DNA isolation kits (MoBio Laboratories, GeneWorks Pty Ltd, South Australia, Australia) in accordance with the manufacturer's instructions. Quant-iT™ dsDNA BR assay kits, a Qubit fluorometer (Life Technologies, Auckland, New Zealand), and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Montchanin, USA), were used to determine the concentration and quality of the extracted DNA. For real-time qPCR analysis, samples were prepared using a CAS-1200 Robotic liquid handling system (Corbett Life Science, BioStrategy, Auckland, New Zealand), and analysed using a Rotor-Gene™ 6000 real-time rotary analyser (Corbett Life Science, BioStrategy, Auckland, New Zealand), using the thermal profile described in Table 5.2.

Table 5.2: Primer pairs and PCR conditions used in real-time qPCR analysis (Di *et al.* 2014).

Target group	Primer name	Sequence (5'-3')	Length of amplicon (bp)	Primer final concentration (nM)	Thermal profile	Amplification efficiency (R^2>0.99) (%)	References
amoA	amoA1F amoA2R	5'-GGGGTTTCTACTGGTGGT-3' 5'-CCCCTCKGSAAAGCCTTCTTC-3'	491	250	95 °C for 2 min - x 1 cycle; 95 °C for 20 s, 57 °C for 30 s, 72 °C for 30 s, 85 °C for 15 s - x 40 cycles;	96-98	(Rotthauwe *et al.* 1997)
nirS	Cd3aF R3cd	5'-GTSAACGTSAAGGARACSGG-3' 5'-GASTTCGGRTGSGTCTTGA-3'	410	750	95 °C for 2 min - x 1 cycle; 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, 85 °C for 20 s - x 40 cycles;	93-95	(Michotey *et al.* 2000, Throback *et al.* 2004)
nosZ	NosZ-F NosZ1622R	5'-CGYTGTTCMTCGACAGCCAG-3' 5'-CGSACCTTSTTGCCSTYGCG-3'	424	750	95 °C for 2 min – x 1 cycle; 95 °C for 20 s, 58°C for 30 s, 72 °C for 30 s, 85 °C for 15 s - x 40 cycles;	94-99	(Kloos *et al.* 2001, Throback *et al.* 2004)

5.2.7 Data analysis

Nitrous oxide emission rates were calculated linearly from the increase in concentration of N_2O between the first and second gas samples taken at each sampling event. Daily N_2O fluxes were then calculated on the assumption that the calculated hourly flux represented the average hourly flux for that day. Total N_2O emissions were calculated by integrating the daily emission fluxes.

The average concentration of NH_4^+, NO_3^-, and abundance of gene copies throughout the duration of the experiment was determined by taking the average of the daily concentration between sampling events.

The emission factor (EF_3), or proportion of applied N that was emitted as N_2O was calculated using Equation 5.2 (de Klein *et al.* 2003):

$$EF(\%) = \frac{N_2O\text{-}N\ total\ (treatment) - N_2O\text{-}N\ total\ (control)}{UrineN\ (applied)}\ x\ 100 \qquad (5.2)$$

where *EF(%)* is the emission factor, *N_2O-N total (treatment)* is the cumulative total N_2O emitted from a urine treatment, *N_2O-N total (control)* is the cumulative total N_2O emitted from the comparative no-urine treatment, and *UrineN (applied)* is the amount of N added as urine.

Least significant differences and P-values were calculated following analysis of variance using the GenStat software package (version 16, VSN International Ltd, UK).

5.3 Results

5.3.1 Nitrous oxide emissions

Peak N_2Oemission flux

In the trampled soil at 50% WFPS (T25 treatment), the hourly N_2O emission flux peak was 0.8 µg N_2O-N kg dry soil^{-1} hr^{-1}, which increased to 1.3 µg N_2O-N kg dry soil^{-1} hr^{-1} with the addition of dairy cow urine (TU25) (Figure 5.5a). In the trampled soil at 80% WFPS (T41), the peak hourly N_2O emission flux was 0.6 µg N_2O-N kg dry soil^{-1} hr^{-1}, which increased to 22.8 µg N_2O-N kg dry soil^{-1} hr^{-1} with the addition of dairy cow urine (TU41). In the soil that was not trampled, at 80% WFPS (NT50), the hourly N_2O emission flux peak was 12.4 µg N_2O-N kg dry soil^{-1} hr^{-1}, which increased to 78.5 µg N_2O-N kg dry soil^{-1} hr^{-1} with the addition of dairy cow urine (NTU50) (Figure 5.5a).

Applying DCD to the soil reduced peak N_2O emissions from the drier, trampled soil, with urine (TU25) by 85%, from 1.3 µg N_2O-N kg dry soil^{-1} hr^{-1} to 0.2 µg N_2O-N kg dry soil^{-1} hr^{-1} (TUD25) (Figure 5.5b). DCD reduced peak N_2O emissions from the trampled, moist soil, with urine, (TU41) by 80%, from 22.8 µg N_2O-N kg dry soil^{-1} hr^{-1} to 4.6 µg N_2O-N kg dry soil^{-1} hr^{-1} (TUD41). DCD reduced peak N_2O emissions from the moist, non-trampled soil, with urine (NTU50), by 73%, from 78.5 µg N_2O-N kg dry soil^{-1} hr^{-1} to 21.6 µg N_2O-N kg dry soil^{-1} hr^{-1} (NTUD50). Peak N_2O emissions from the TD25 treatment were 0.8 µg N_2O-N kg dry soil^{-1} hr^{-1} (Figure 5.5b).

Total N_2O emissions

In the trampled soil at 50% WFPS (T25), the total N_2O emission flux over the experimental period was 0.8 mg N_2O-N kg dry soil^{-1}, which increased to 1.6 mg N_2O-N kg dry soil^{-1} with the addition of dairy cow urine (TU25) (Table 5.3). In the trampled soil at 80% WFPS (T41), the total N_2O emission flux was 0.6 mg N_2O-N kg dry soil^{-1}, which increased to 29.4 mg N_2O-N kg dry soil^{-1} with the addition of dairy cow urine (TU41). In the soil that was not trampled, at 80% WFPS (NT50), the total N_2O emission flux was 4.5 mg N_2O-N kg dry soil^{-1}, which increased to 99.1 mg N_2O-N kg dry soil^{-1} with the addition of dairy cow urine (NTU50) (Table 5.3). Surprisingly, the amount of N_2O emitted by the un-trampled (NTU50) soil was significantly higher than that from the trampled (TU41) soil ($P<0.05$).

Applying DCD to the soil reduced total N_2O emissions from the drier, trampled soil, with urine (TU25) by 77% to 0.4 mg N_2O-N kg dry soil^{-1} (TUD25); from the moist, tramped soil, with urine (TU41), by 91% to 2.7 mg N_2O-N kg dry soil^{-1} (TUD41); and from the moist, non-trampled soil, with urine (NTU50) by 87% to 13.1 mg N_2O-N kg dry soil^{-1} (NTUD50). Total N_2O emissions from the TD25 treatment were 0.76 mg N_2O-N kg dry soil^{-1} (Table 5.3).

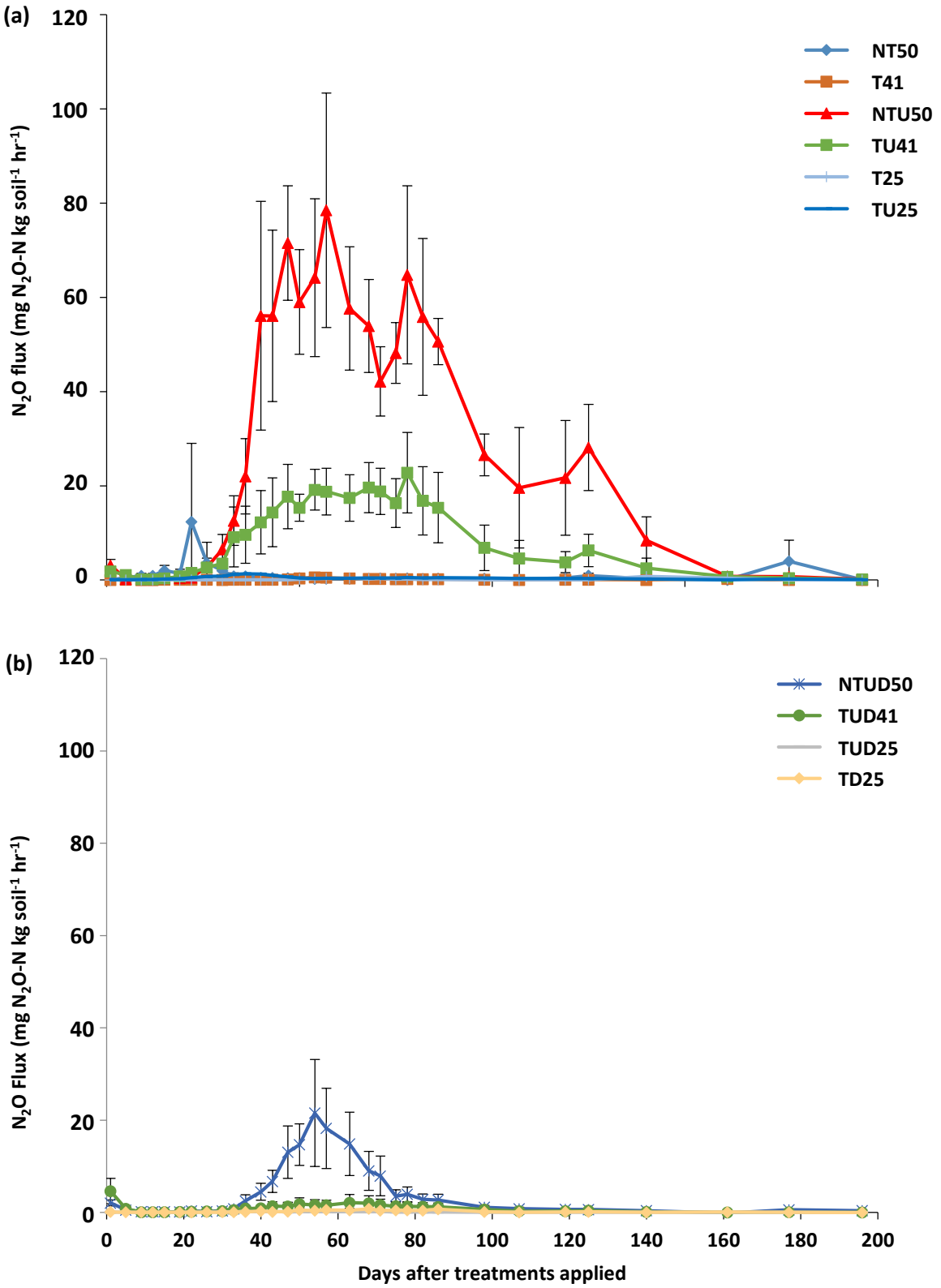

Figure 5.5: **Daily N_2O emission rates from (a) non-DCD, and (b) DCD amended treatments. Error bars represent one standard error of the mean.**

Table 5.3: Volumetric water content, water filled pore space, total cumulative N_2O emissions, reduction in N_2O emissions following DCD application, and proportion of applied N emitted as N_2O during the period 8th February to 22th August 2012.

Treatment	θv (v/v%)	Water filled pore space (%)	Total emissions (mg N_2O-N kg $soil^{-1}$)	Reduction (%)	Emission factor (% of applied N)
T25	25	50	0.8	-	-
TU25	25	50	1.6	-	0.14
TUD25	25	50	0.4	77	-0.07
TD25	25	50	0.7	12	-
T41	41	80	0.6	-	-
TU41	41	80	29.4	-	4.94
TUD41	41	80	2.7	91	0.36
NT50	50	80	4.5	-	-
NTU50	50	80	99.1	-	16.23
NTUD50	50	80	13.1	87	1.48
LSD# (0.05)	-	-	23.0	-	-

#Least significant difference (P < 0.05).

5.3.2 Soil mineral N

Ammonium

The average NH_4^+ concentration in the soil of all the urine amended treatments (without DCD) one day after urine application was 481.3 mg NH_4-N kg $soil^{-1}$, and did not change significantly for at least 21 days (Table 5.4, Figure 5.6a). After 21 days, the concentration of NH_4^+ within the soil of the TU25, TU41, and NTU50 treatments began to decline. The concentration of NH_4^+ within the soil of the NTU50 treatment reached background levels after 92 days, while the higher density TU25, and TU41 treatments took up to 212 days before the NH_4^+ concentration reached background levels (Figure 5.6a).

The concentration of NH_4^+ in the soil of the treatments that received DCD did not change significantly throughout the duration of the experiment, with the exception of the NTUD50 treatment, where the NH_4^+ concentration decreased by about 45% during the final 71 days of incubation (Figure 5.6b). However, the NH_4^+ concentration was, on average, higher in the TUD25 soil than it was in the TUD41 soil ($P<0.05$), which was higher than the average NH_4^+ concentration in the NTUD50 soil ($P<0.05$) (Table 5.4).

The NH_4^+ concentration in the soil of the NT50, T41, and T25 treatments where urine was not applied were not significantly different throughout the duration of the experiment. The concentration of NH_4^+ in the TD25 treatment, however, increased from 7.5 mg NH_4-N kg $soil^{-1}$ to 27.5 mg NH_4-N kg $soil^{-1}$ during the experimental period (Figure 5.8).

Nitrate

The average NO_3^- concentration in the soil of the urine amended treatments (without DCD) one day after urine application was 15.3 mg NO_3-N kg $soil^{-1}$ (Figure 5.7a). Nitrate accumulated in the soil of the NTU50 treatment, peaking at 443.3 mg NO_3-N kg $soil^{-1}$ after 141 days of incubation. A similar NO_3^- accumulation was seen in the TU25 treatment where the NO_3^- concentration peaked at 522.6 mg NO_3-N kg $soil^{-1}$ after 212 days of incubation (Figure 5.7a). However, the concentration of NO_3^- in the TU41 treatment decreased from 15.1 mg NO_3-N kg $soil^{-1}$ to <0.1 mg NO_3-N kg $soil^{-1}$ after 8 days of incubation, and did not significantly exceed the initial concentration for 92 days, when a concentration of 25 mg NO_3-N kg $soil^{-1}$ was observed (Figure 5.7a). After 92 days of incubation, the concentration of NO_3^- in the TU41 treatment soil increased until a concentration of 225 mg NO_3-N kg $soil^{-1}$ was detected at the completion of the experiment (Figure 5.7a).

The average NO_3^- concentration in the TU25 soil was higher than the NTU50 soil ($P<0.05$), which was higher than the average NO_3^- concentration in the TU41 soil ($P<0.05$) (Table 5.4).

Applying DCD to the soil in combination with cow urine reduced the NO_3^- concentration in all three soil moisture/compaction treatments throughout the duration of the experiment (Figure 5.7b).

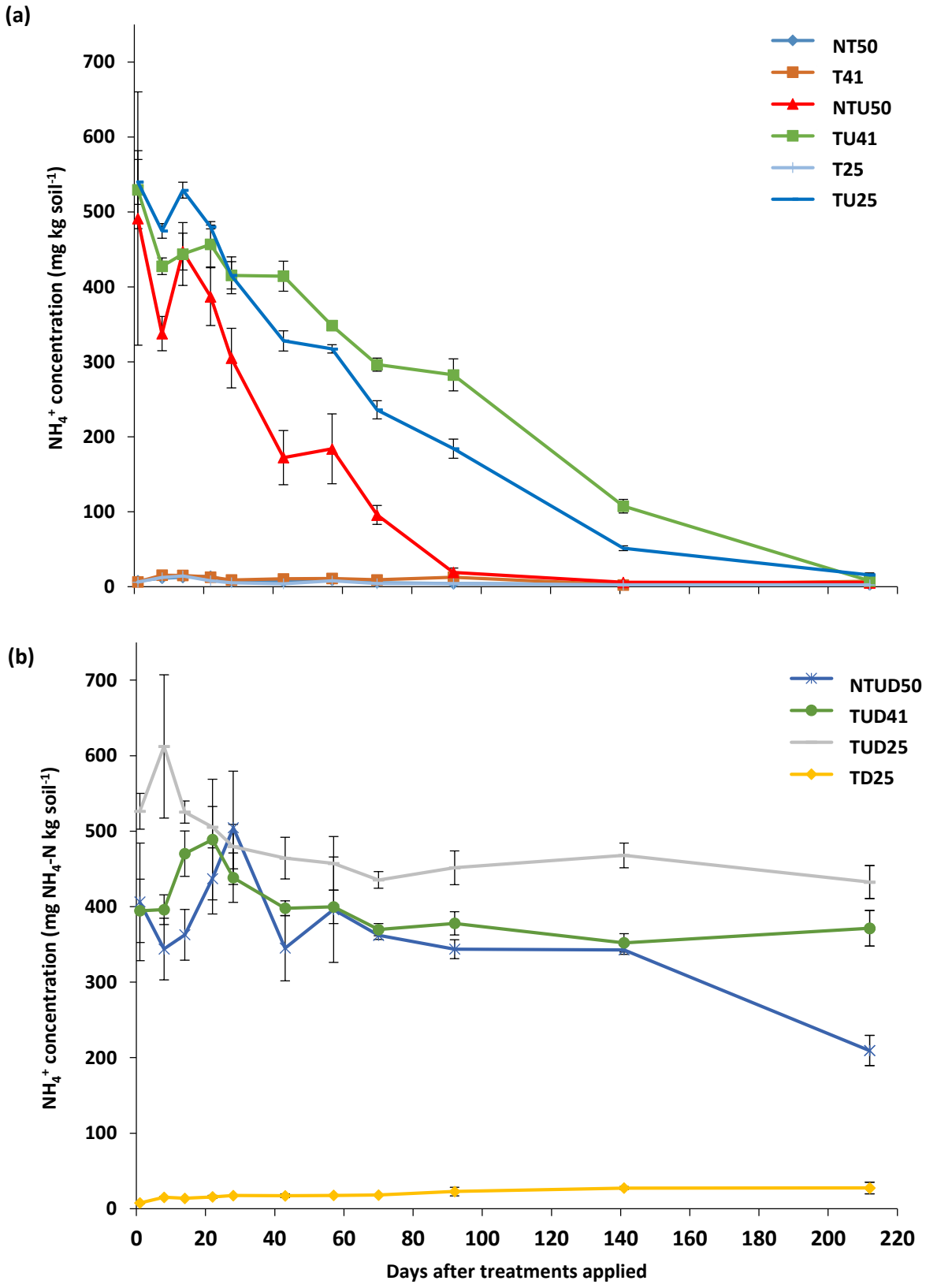

Figure 5.6: **Ammonium concentration in the soil of the urine affected treatments (a) without DCD, and (b) with DCD during the period from 9th February to 7th September 2012. Error bars represent one standard error of the mean.**

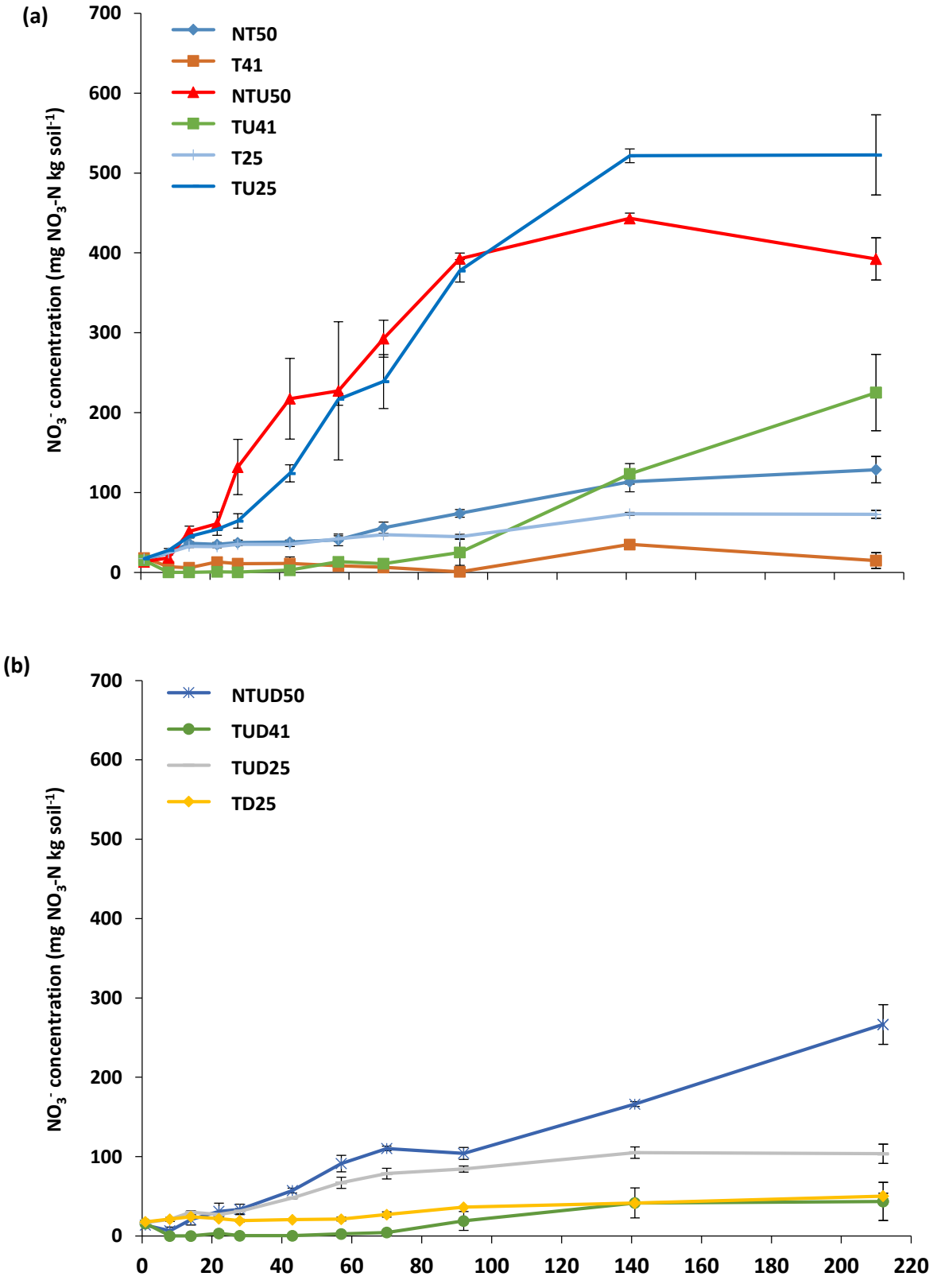

Figure 5.7: **Nitrate concentration in the soil of the treatments (a) without DCD, and (b) with DCD during the period from 9th February to 7th September 2012. Error bars represent one standard error of the mean.**

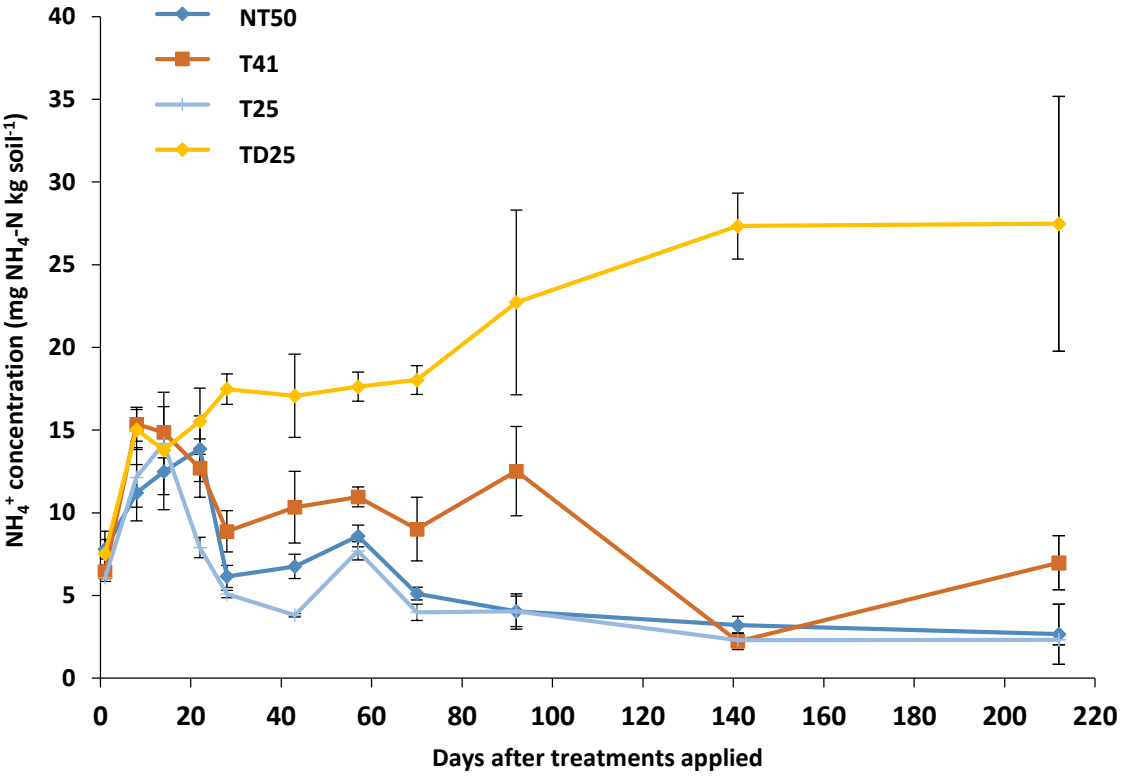

Figure 5.8: **Ammonium concentration in the soil of the treatments that did not receive dairy cow urine, from 9th February to 7th September 2012. Error bars represent one standard error of the mean.**

Table 5.4: **Average mineral nitrogen concentration in the soil over the duration of the experiment, from 9th February to 7th September 2012.**

Treatment	Average NH_4^+ (mg NH_4-N kg $soil^{-1}$)	Average NO_3^- (mg NO_3-N kg $soil^{-1}$)
T25	4.3	54.5
TU25	188.3	349.6
TD25	22.4	34.8
TUD25	464.7	80.0
T41	7.82	16.0
TU41	227.12	79.4
TUD41	382.1	23.32
NT50	5.2	81.4
NTU50	98.6	323.0
NTUD50	338.6	132.3
LSD# (0.05)	18.6	19.6

#Least significant difference ($P < 0.05$).

5.3.3 DCD concentration

The concentration of DCD in the soil remained reasonably constant in all treatments for the first three months of incubation, but declined towards the end of the experiment (Figure 5.9). The soil in the moist, trampled soil with urine (TUD41) had a higher average DCD concentration than the non-trampled (NTU50) and drier trampled (TUD25) soils ($P<0.05$). The concentration of DCD in the soil without urine (TD25) had the lowest average DCD concentration ($P<0.05$).

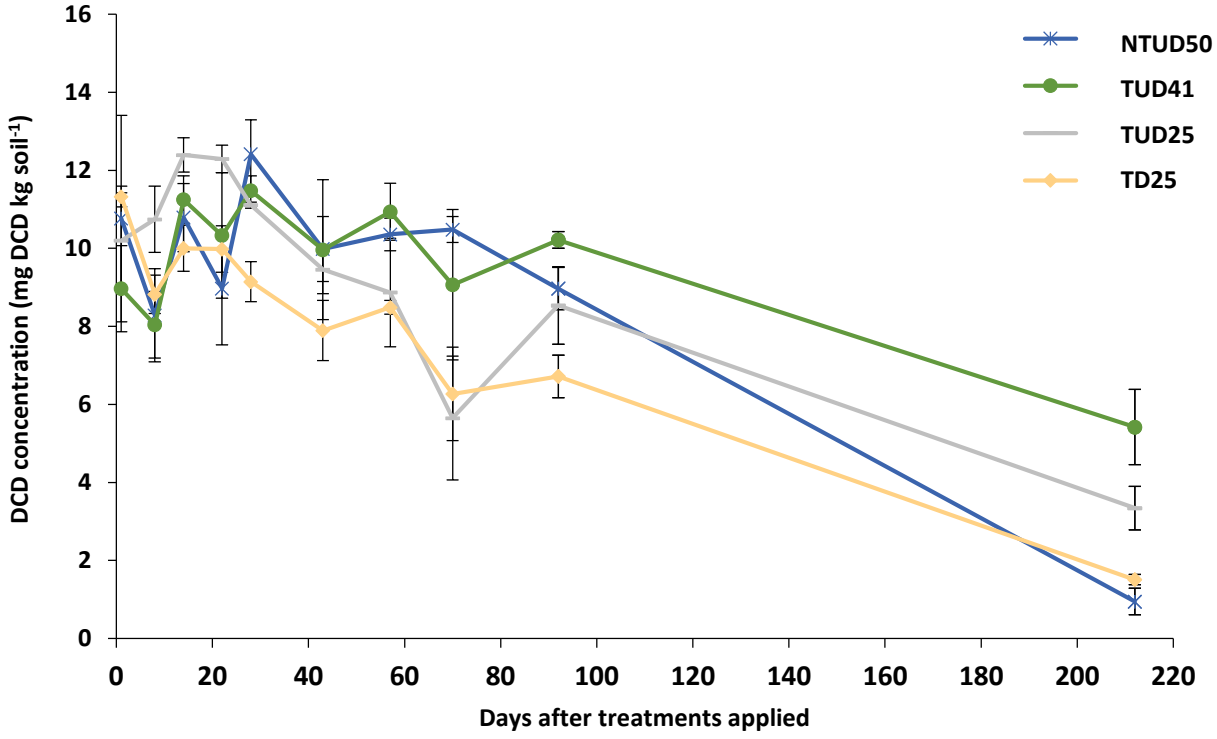

Figure 5.9: **Concentration of DCD in the soil during the period from 9th February to 7th September 2012. Error bars represent one standard error of the mean.**

5.3.4 Incubation method comparison

Soil was incubated in two different types of vessels throughout the experiment to provide un-disturbed soil for analysis. The soil for N_2O emission calculations was incubated in 575 ml glass jars, while the soil for the destructive mineral nitrogen, and microbial assay, was incubated in 50 ml plastic centrifuge tubes. At the completion of the experiment, the mineral nitrogen concentration in the soil of each of the 40 soil-filled jars, and 40 soil filled tubes, was assessed to compare the effect of the two methods.

No significant difference was found in the NH_4^+ concentration in the soil of any of the treatments between the two incubation methods, except for the dry, trampled soil with urine (TU25),

where the average NH_4^+ concentration in the tubes was lower than that in the glass jars (p=0.027) (Table 5.5).

The concentration of NO_3^- was lower in the tubes, than it was in the jars, for the trampled soil at the higher soil moisture without urine (T41) (p<0.001), with urine (TU41) (p=0.037), and with urine and DCD (TUD41) (p=0.008).). However, there was no significant difference found in the NO_3^- concentration in the soil between the two incubation methods of the remainder of the treatments (Table 5.6).

Table 5.5: Comparison of the concentration of NH_4^+ in the soil incubated in centrifuge tubes, with the concentration of NH_4^+ in soil stored in jars, at the completion of the experiment.

Treatment	NH_4-N in tubes (mg NH_4-N kg $soil^{-1}$)	NH_4-N in jars (mg NH_4-N kg $soil^{-1}$)	LSD# (0.05)
T25	2.32	3.90	1.61
TU25	15.6	33.7	15.26
TUD25	432	394	69.3
TD25	27.5	33.4	19.5
T41	6.98	3.64	4.12
TU41	7.65	5.68	4.356
TUD41	371	346	68.3
NT50	3.31	3.66	3.133
NTU50	5.29	5.42	1.819
NTUD50	209	173	83

#Least significant difference (P < 0.05).

Table 5.6: Comparison of the concentration of NO_3^- in the soil incubated in centrifuge tubes, with the concentration of NO_3^- in soil stored in jars, at the completion of the experiment.

Treatment	NO_3-N in tubes (mg NH_4-N kg $soil^{-1}$)	NO_3-N in jars (mg NH_4-N kg $soil^{-1}$)	LSD (0.05)
T25	72.6	84.2	13.7
TU25	523	544	196
TUD25	103.6	106	32.4
TD25	50.2	50.4	12.2
T41	14.8	161.9	48.78
TU41	225	406	165.8
TUD41	44	161	73.6
NT50	129	163	58.5
NTU50	329	351	111.4
NTUD50	267	244	85.9

#Least significant difference ($P < 0.05$).

5.3.5 Microbial gene abundance

The abundance of NO_2^- reducing *nirS* gene copies were, on average, higher in the soil of the TU41 treatment than in both the NTU50 and TU25 treatments ($P<0.05$) (Figure 5.10a). The abundance of N_2O reducing *nosZ* gene copies were, on average, higher in the TU41 soil than in the TU25 soil ($P<0.05$), but not significantly different from the NTU50 soil (Figure 5.10b). The average abundance of the bacterial NH_4^+ oxidising *amoA* gene copies were generally higher in the un-compacted soil (NTU50), however the differences between treatments were, on average, not significantly different. The second sampling period was the only time during the experiment when a significant difference between treatments ($P<0.05$) was observed, when *amoA* gene copies were higher in the un-trampled soil (NTU50) than the trampled treatments (TU25, TU41) (Figure 5.11).

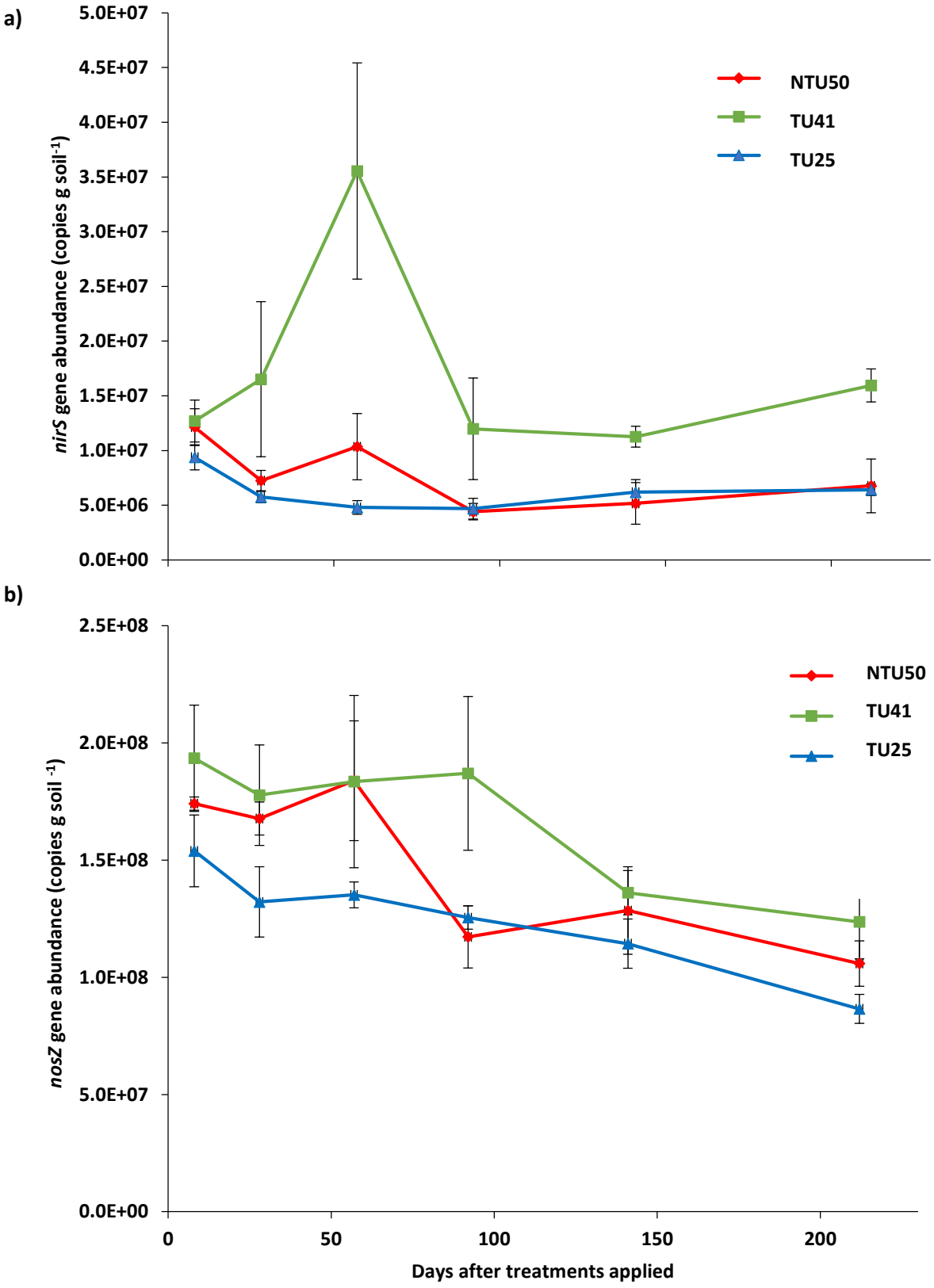

Figure 5.10: **Abundance of denitrifying (a) NO_2^- reductase *nirS,* and (b) N_2O reductase *nosZ* gene copies in the soil during the period from 9th February to 7th September 2012. Error bars represent one standard error of the mean.**

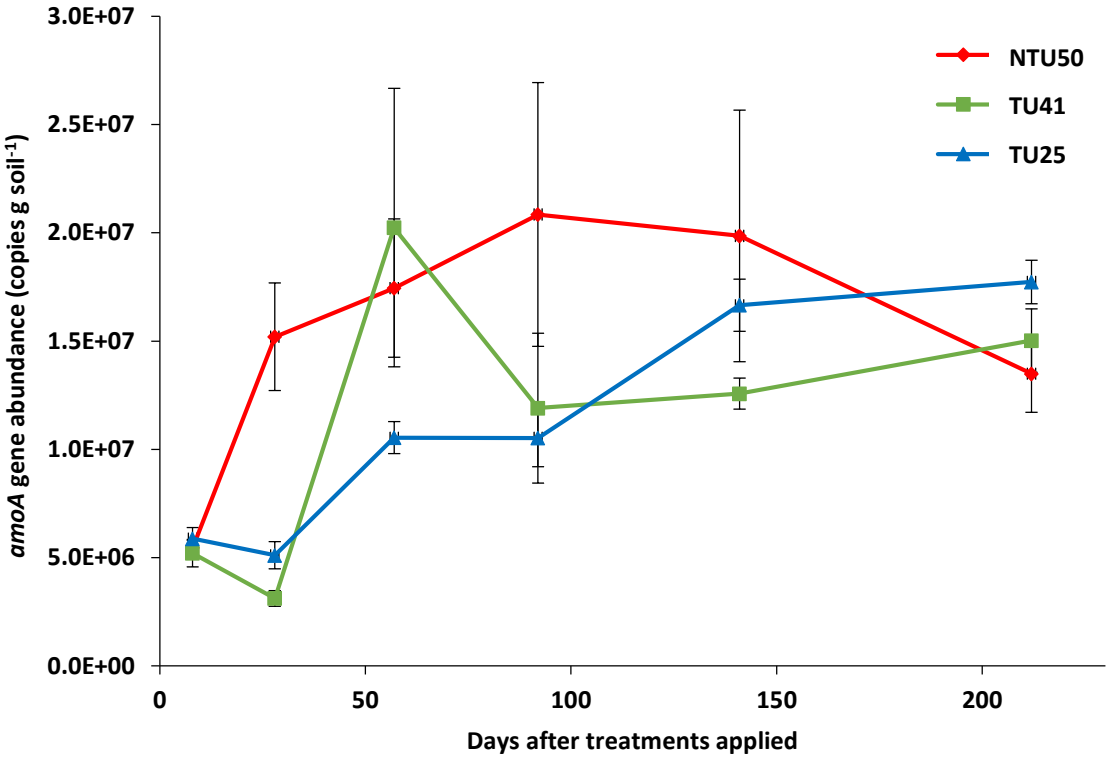

Figure 5.11: **Ammonia oxidising bacterial *amoA* gene copy numbers in the soil during the period from 9th February to 7th September 2012. Error bars represent one standard error of the mean.**

5.4 Discussion

Previous work has shown that the combination of urine-N, and wet, compacted soil, are an ideal environment to generate large N_2O emissions (e.g. van Groenigen *et al.* 2005a, Bhandral *et al.* 2007, Ball *et al.* 2012). However, results presented here are contradictory, with total N_2O emissions following the addition of cow urine to un-compacted soil being more than three times higher than those from compacted soil.

There are several approaches to quantifying the moisture status of soil, and WFPS has often been cited as being positively correlated with N_2O emission (e.g. Weier *et al.* 1993, Dobbie *et al.* 1999, Ball *et al.* 2008). When kept at a constant bulk density, changing the WFPS of a soil will produce a proportional change in volumetric water content (θ_v). However, when the WFPS of a soil remains constant, and the density of the soil changes, there will be a corresponding change in θ_v. Consequently, in this study, the un-trampled soil at 80% WFPS had a θ_v of 50%, the trampled soil at 80% WFPS a θ_v of 41%, and the trampled soil at 50% WFPS a θ_v of 25%. When considering the WFPS of the soils in the present experiment, the results apparently contradict those of other authors (e.g. Davidson 1993, van Groenigen *et al.* 2005a) who found N_2O emissions increasing as WFPS increased. However, when using the θ_v as a measure of soil moisture, the results presented here are in agreement with those of Di *et al.* (2014), who showed that N_2O emission from urine affected soil increased as θ_v increased, and van der Weerden *et al.* (2012a) who concluded that θ_v better explained N_2O emissions from soil than WFPS. The results of the present study demonstrate that factors other than WFPS need to be considered when researching the drivers of N_2O emissions from soil.

The loosely packed non-trampled soil in the present study provided a continuous network of large pores that was not present in the trampled soil (Figure 5.3), and this continuous pore network in the non-trampled soil would have enabled N_2O to easily diffuse out of the soil (Renault and Stengel 1994). In comparison, the compacted nature of the trampled soils would have inhibited N_2O diffusion to the atmosphere. The inability of N_2O to exit the trampled soil may have reduced the total N_2O emission flux, but not necessarily the production of N_2O within the soil.

The porous structure of the non-trampled soil may have also allowed oxygen to diffuse easily into the soil, and when combined with adequate soil moisture, may have allowed high rates of mineralisation and nitrification to occur on the periphery of soil aggregates. This hypothesis is supported by the fact that there was a comparatively rapid decline in soil NH_4^+ concentration in the non-trampled soil (NTU50) (Figure 5.6a), which suggests the rate of nitrification was highest in the non-trampled soil. Furthermore, at the completion of the experiment, the NO_3^- concentration the non-trampled soil when no urine was added (NT50) was 129 mg NO_3-N kg $soil^{-1}$ (Figure 5.7a), an increase from 16 mg NO_3-N kg $soil^{-1}$ at the start of the experiment, indicating mineralisation of the native soil N

was occurring. Nitrifier denitrification in the aerobic, non-trampled soil may therefore have contributed to the large N_2O emission flux, as reported by others (e.g. Robertson and Tiedje 1987, Wrage *et al.* 2001).

More denitrifying gene copies were found in the moist, trampled soil (TU41), than in the other treatments (NTU50 and TU25). On average, copies of the NO_2^- reductase *nirS* gene were significantly ($P<0.05$) more abundant in the TU41 soil than in both the NTU50 and TU25 soil, and copies of the N_2O reductase *nosZ* gene were significantly ($P<0.05$) more abundant in the TU41 soil than in the drier TU25 soil (Figure 5.10a,b). The increase in abundance of denitrifying gene copies validates the hypothesis that complete denitrification was favoured in the trampled, moist soil, compared to the drier and non-compacted soils. Higher numbers of *nosZ* gene copies would imply that more N_2O was reduced to N_2 (Groffman *et al.* 2006), which may help to partially explain why the TU41 soil emitted less N_2O than the NTU50 soil. Furthermore, Chapuis-Lardy *et al.* (2007), and Fowler *et al.* (2009) concluded that N_2O can be consumed by denitrifiers within soil if gas diffusion is restricted (such as in the trampled soil). Unfortunately, N_2 emissions were not measured in this experiment.

When urine was applied, the apparent nitrification rate was considerably different between non-trampled (NTU50) and trampled (TU41) treatments. The concentration of NH_4^+ decreased much more rapidly in the non-trampled soil than in the trampled soil, indicating more rapid nitrification was happening (Figure 5.8a). Concomitantly, the concentration of NO_3^- increased much more rapidly in the non-trampled soil than it did in the trampled soil (Figure 5.7a). On average, the abundance of bacterial ammonia oxidising (*amoA*) gene copy numbers were higher in the non-trampled soil than the other treatments, though the averages were not significantly different. Only at one sampling point (28 days after treatments applied, Figure 5.11) were *amoA* copy numbers higher ($P<0.05$) in the non-trampled soil than other treatments. The high variability observed in *amoA* gene copy numbers in the non-trampled soil (Figure 5.11) makes the statistical comparison to the other treatments non-significant.

A proportion of the N applied as urine may have been volatilised as NH_3 (Hristov *et al.* 2011). However, the cool, moist conditions of the present experiment did not favour volatilisation (Cameron *et al.* 2013), and Bussink and Oenema (1998) found that only 6% of urine N was volatilised as NH_3, therefore losses of N through volatilisation of NH_3 from any of the treatments in the present study are unlikely to be high. Additionally, in this study, the NH_4^+ concentration in treatments with urine, and the nitrification inhibitor, dicyandiamide (DCD), did not change significantly throughout the experimental period (Figure 5.6b), demonstrating NH_3 volatilisation losses were probably low, even when soil NH_4^+ concentration was high.

In a field study, de Klein *et al.* (2011) observed that the application of dairy cow urine applied to un-trampled soil in Canterbury increased total N_2O emissions by 25 times, which was comparable

to increases seen in the TU41, and NTU50 treatment in the present study. Similarly, N_2O emission increased by 25 times when urine was added to trampled soil in Chapter Four of this thesis.

The emission factor (EF_3) or proportion of applied N emitted as N_2O by the TU25 soil was 0.1% which was slightly below the 0.2-6.2% range of EF_3 values reported by de Klein *et al.* (2003), while the EF_3 of the TU41 soil was 4.9% (Table 5.3), which was within the reported range. At 16.2% of applied N, the EF_3 value of the NTU50 soil was considerably higher than observed by other authors reporting EF_3 values from field studies (e.g. Clough *et al.* 1998, Di and Cameron 2008, Ball *et al.* 2012). However, in other laboratory studies, de Klein and van Logtestijn (1994) and Lovell and Jarvis (1996) saw up to 15-16% of applied N lost as N_2O over comparatively short time periods. While the EF_3 of the NTU50 soil reported in the present study was similar to those reported by de Klein and van Logtestijn (1994) and Lovell and Jarvis (1996), in a review, the average EF_3 from 13 incubation experiments was 2.1% (van Groenigen *et al.* 2005a). The highest EF_3 recorded in the field conditions of Chapter Four of this thesis was 1.9%, which was considerably less than the values for the more moist soils (TU41 & NTU50) in the current laboratory study. The lack of drainage and leaching of N, and lack of plant uptake of N in the incubation vessels in the present experiment created an artificial environment not seen in the field, leading to elevated soil N concentrations, and can probably account for the higher EF_3 values seen here.

Although the temporal pattern of N_2O emissions was remarkably similar between the TU41 and NTU50 treatments (Figure 5.5a), the mineral N concentrations in the soil differed considerably. The concentration of NO_3^- in the TU41 soil did not increase significantly during the first 92 days of incubation (Figure 5.7a), which suggests the rate of denitrification was exceeding the rate of nitrification. The concentration of NH_4^+ in the TU41 soil decreased by about 50% over the same 92 day period, which indicates nitrification was indeed taking place. In the TU41 soil, the declining concentration of NH_4^+ coupled with a lack of significant increase in the concentration of NO_3^-, indicates that the NO_3^- was denitrified as soon as it was produced, signalling that the rate of denitrification in the TU41 soil may have initially been limited by the supply of NO_3^- (Myrold 1998).

Applying the nitrification inhibitor, dicyandiamide (DCD) together with the urine, created substantial reductions in N_2O emissions. Total N_2O emission were reduced by 77% when applied to the dry, trampled soil (TU25), by 87% when applied to the moist, non-trampled soil (NTU50), and by 91% when applied to the trampled, moist soil (TU41) (Table 5.3). The reductions presented here are higher than those reported in a similar incubation study using wet soil, where N_2O emission was reduced by 44-68% (Di *et al.* 2014). The reductions are also higher than the average of 57% from field studies as reviewed by de Klein *et al.* (2011). However, the reductions are similar to the 82-86% shown by Di and Cameron (2002, 2003) who used a comparative stony soil from Canterbury. The cool incubation temperature of the present experiment (10°C) would have maximised the effectiveness of

DCD to reduce N_2O emissions, leading to the high N_2O flux reductions reported here. The average half-life of DCD in treatments of this study was between 92 and 212 days (Figure 5.9), which is in agreement with (Di and Cameron 2004a), who found that at 8°C, the half-life was of DCD was about 100-116 days. The half-life of DCD in the present experiment was slightly more than the estimate of Kelliher *et al.* (2008), who calculated that DCD in soil at 10°C would have a half-life of about 72 ± 14 days.

On average, the concentration of DCD was higher ($P<0.05$) in the moist, trampled soil (TUD41) than in the drier, trampled soil (TU25), and in the non-trampled soil (NTUD50) (Figure 5.9). The soil with DCD but no urine-N (TD25) had the lowest average DCD concentration ($P<0.05$), which is in agreement with Kelliher *et al.* (2008), who suggests that microbes in N limited soil may mineralise the N contained within DCD at a faster rate than in soil with surplus N.

Under field conditions, the rapid accumulation of NO_3^- in the non-trampled soil would be expected to leach through the soil and be unavailable for denitrification in the soil profile. In comparison, the slower conversion of NH_4^+ through to NO_3^- in tramped soil would be likely to produce more N_2O via denitrification over a longer time period. In addition, wetting and drying cycles experienced under field conditions, similar to that seen in Chapter Four of this thesis, have been shown to cause spikes in N_2O emission fluxes (e.g. Ball *et al.* 2012). Given that the key objectives of this thesis focused on denitrification and losses of N_2O from winter grazed dairy forage soil at field scale, subsequent experiments in this thesis were field based. The air permeability of the non-trampled soil may have led to the un-expectedly high N_2O emissions observed in the present experiment, and air permeability was therefore investigated in the next set of experiments in this thesis. As this laboratory experiment was run concurrently with the second field experiment (Chapter Six), some aspects requiring further investigation – such as measuring N_2 loss, and increasing the number of treatment replicates – were not included until the third field experiment (Chapter Seven).

5.5 Conclusions

The application of a nitrification inhibitor, DCD, reduced N_2O emissions from urine affected soil in this study by 77-91%. When dairy cow urine was present, N_2O emissions were positively correlated with the volumetric water content of the soil, where soil with a θ_v of 50% released more than three times as much N_2O as soil with a θ_v of 41%.

The un-compacted soil featured a large connected pore network that was not evident in the compacted soil. These large pores may have allowed a free gaseous exchange into, and out of the soil, which may have enhanced nitrifier denitrification. The hypothesis of enhanced nitrifier denitrification is supported by the differences in soil ammonium and nitrate measured in the different treatments.

The wet, trampled soil had higher denitrifying gene copy numbers compared to the other treatments, which strongly suggests that more complete denitrification was occurring in the wet, trampled soil than in the non-trampled soil. Complete denitrification may have resulted in the apparent difference in N_2O emissions from the treatments.

Chapter Six – The effect of simulated winter animal grazing of forage crops, and the nitrification inhibitor, dicyandiamide, on nitrous oxide emissions from soil – a field experiment

6.1 Introduction

Nitrous oxide, a powerful greenhouse gas (GHG), has reached atmospheric concentrations not seen during the past 800,000 years (IPCC 2013). Direct and indirect emissions from animal excreta deposited onto soil (Figure 6.), are responsible for the majority of NZ's nitrous oxide (N_2O) emissions (Ministry for the Environment 2013). The number of dairy cows in the Canterbury region of New Zealand has increased substantially in recent years, and the majority of cows live outside all year. Dairy herds are taken to graze high yielding forage crops during the winter, which can lead to animal trampling damage and concentrated urine deposition during the cool, wet, conditions (Drewry and Paton 2005) (Figure 6.1, Figure 6.2). Both urine deposition and animal trampling produce soil conditions conducive to denitrification, and elevated N_2O emissions (Menneer *et al.* 2005, van Groenigen *et al.* 2005b, Ball *et al.* 2012).

Figure 6.1: **A dairy cow urinating on recently grazed and heavily trampled soil, at Lincoln University's Ashley Dene Research farm during winter, 2012.**

Production of N_2O in an agricultural setting mostly occurs during the microbial processes of nitrification and denitrification (Firestone and Davidson 1989, Groffman *et al.* 2006). In Chapter Five of this thesis, it was reported that significantly more copies of denitrifying microbial genes were found in the trampled, moist soil, than were found in drier or un-compacted soil, which is supported by Di *et al.* (2014), who found a positive relationship between soil moisture and denitrifying gene abundance. However, no authors have reported changes in denitrifying microbial gene copies in winter forage grazing soils, or their relationship to N_2O emissions.

Ball *et al.* (2012) found N_2O emissions increased when dairy pasture was subject to compaction, and they also found the nitrification inhibitor, DCD, reduced those N_2O emissions by as much as 63%. Similarly, van der Weerden *et al.* (2012b) saw a reduction of 71% in N_2O emissions when DCD was applied to compacted soil in a forage system. Nitrous oxide emissions from grazed forage crops in the South Island of New Zealand have been studied in fine textured, pallic soil (Smith *et al.* 2008b, Thomas *et al.* 2008, van der Weerden *et al.* 2012b), but not in stony soil, as commonly found in Canterbury. More information on N_2O emissions from forage crops is needed, as the Ministry for the Environment (2011) has concluded that there is insufficient information available to calculate national N_2O emissions from dairy cows grazing forage crops during winter.

The objectives of this study were to determine the effects of animal trampling and the effectiveness of DCD on N_2O emissions, soil N concentrations, and denitrifying microbial gene abundance in stony soil under simulated winter grazing conditions in the field. In addition, changes in the bulk density and air permeability of the soil following simulated animal trampling were investigated.

Figure 6.2: **Wet, trampled soil, and high animal stocking rates combine to provide ideal conditions for denitrification to occur when dairy cows graze forage crops in winter.**

6.2 Methods

6.2.1 Soil and climate

Soil and lysimeter construction

A Balmoral stony silt loam, classified as Pallic Orthic Brown Soil (Hewitt 2010); Udic Haplustept loamy skeletal (Soil Survey Staff 2010), typical of soils used for dairy winter forage grazing in the Canterbury region, South Island of New Zealand, was used in this study. Developed from gravelly glacial outwash alluvium and loess, the Balmoral soil consists of a thin, sandy silt loam top soil containing about 30% stones. Below the top soil, the stone and sand content increases substantially, making this soil very free draining (Figure 6.4). When dairy cows graze forage crops grown in the Balmoral soil, considerable structural damage can occur (Figure 6.3).

Figure 6.3: **Extensive damage to the structure of the stony Balmoral soil from dairy cows grazing a forage crop during winter, at Lincoln University's Ashley Dene Research Farm.**

Thirty two lysimeters were collected from un-grazed sections of a Balmoral soil at Lincoln University's Ashley Dene farm (43°38'55"S, 172°20'39"E), during December 2011, using the method described by Cameron et al. (1992). To create the undisturbed soil monoliths, steel casings (50 cm diameter by 70 cm depth) were pushed into the soil in small increments, while the surrounding soil was gently scraped away, leaving an undisturbed soil column within the casing. Any stones that impeded the process were removed from the soil. The edges of the soil column were sealed to the casing with molten petroleum jelly, to prevent edge flow effects, before a cutting plate was inserted beneath the base of the lysimeter housing. The lysimeters were then installed in a lysimeter facility at Lincoln University (43°39'04"S, 172°28'03"E), level with the surrounding soil. The outside of the monoliths were back-filled to maintain the climatic conditions of the surrounding field.

Figure 6.4: **Lysimeters during construction at Ashley Dene farm. Note high stone content throughout soil profile.**

In addition to the 32 lysimeters, 42 soil blocks were established in a facility adjacent to where the lysimeters were contained (Figure 6.5). Ten soil blocks were set aside for physical measurements, and the remaining 32 companion soil blocks enabled destructive soil sampling, while simultaneously, measurements of N_2O emissions were taken from the undisturbed soil in the lysimeters.

To construct the facility where the soil rings were placed, the top soil was removed from an area approximately seven by four meters, and replaced with sand. A concrete nib was formed around the perimeter of the sand pad, and more sand added to bring the level of the sand above that of the surrounding field. The sand provided free draining conditions similar to the Balmoral's stony sub-soil (Figure 6.5). Forty two metal rings, 500 mm in diameter, by 150 mm deep, were arranged on the sand, and were backfilled with soil. Vegetation was removed and fresh soil was collected from a site at Lincoln University's Ashley Dene farm adjacent to where the lysimeters were collected, and placed in each of the soil rings (Figure 6.5). The soil was packed to a similar density to that in the field.

The pasture on the lysimeters was sprayed with herbicide during February 2012, before established kale plants (*Brassica oleracaea* cv. Regal) were translocated from Lincoln University's Ashley Dene Farm. The kale was transplanted with minimal disturbance to the soil. The kale was planted in each lysimeter and soil ring and allowed to reach maturity (Figure 6.5). The mature kale was harvested on 19th June 2012 to simulate grazing, prior to applying the treatments to the lysimeters.

Figure 6.5: Construction of soil rings for destructive soil sampling (left), with mature kale plants growing in soil rings prior to harvest (right).

Climate

The average annual rainfall in the Canterbury region is about 650 mm, and the average annual temperature about 12.1°C. Water input to the lysimeters was maintained at the 75th percentile of the regional average using simulated rainfall when required. An amount of water equal to that supplied to the lysimeters was manually applied to the soil blocks each week. Rainfall at the

experimental site was measured by a tipping bucket rain gauge (model OSK 15180-T, Ogawa Seiki, Japan), while soil and air temperature was measured by Campbell Scientific CS107 sensors at the same facility.

6.2.2 Treatments

Eight treatments (Table 6.1), each with four replicates, were randomly allocated to the lysimeters on 21st June 2012. Following harvest of the kale, DCD was dissolved in water and applied to the trampled + DCD (TD), non-trampled + DCD (NTD), trampled + urine + DCD (TUD), and non-trampled + urine + DCD (NTUD) treatment lysimeters and companion soil blocks, at a rate equivalent to 20 kg ha^{-1} using hand-held sprayers. In accordance with industry best practice, 10 mm of simulated rainfall was applied to the soil following DCD application. The soil in all trampled lysimeters and soil blocks was then trampled using cow hoof simulation equipment (Figure 6.6, Figure 6.7). The hoof simulation equipment comprised a commercially available post hole compactor, with a groove cut in the lower surface to mimic the shape of a cow hoof. A foot peg was welded to the shaft of the trampling device to allow the user to apply force to the shaft.

Table 6.1: Details of the treatments applied to the soil.

Treatment name[#]	Trampling (yes/no)	Urine-N (kg N ha^{-1})	DCD (kg DCD ha^{-1})
T	Y	0	0
TD	Y	0	20
TU	Y	400	0
TUD	Y	400	20
NT	N	0	0
NTD	N	0	20
NTU	N	400	0
NTUD	N	400	20

[#] 'T' indicates simulated trampled treatments, 'NT' indicates no simulated trampling, 'U' indicates urine was applied, 'D' indicates DCD was applied.

Figure 6.6: **Cow hoof simulation device with groove to mimic cow hoof (left), and foot peg for the user to apply force (right).**

The surface of the soil of the T, TD, TU, and TUD treatments were trampled for a period of 5 minutes per 0.2 m^2 lysimeter or soil ring, at which point the soil visually resembled field observations of soil where cows had grazed forage crops during winter (Figure 6.7).

Fresh urine was collected from non-lactating dairy cows that were feeding on kale, and analysed for total N concentration using an Elemental Analyser (Elementar Vario MAX CN). The urine was standardised to 4.0 g N L^{-1} by adding a small amount of urea, and 2 litres was applied to the TU, NTU, TUD, and NTUD lysimeters and soil blocks, at a rate equivalent to 400 kg N ha^{-1} (in Chapter Four of this thesis, urine was applied at a rate equivalent to 700 kg N ha^{-1}, however subsequent analysis of the urine from cows grazing kale indicated that 400 kg N ha^{-1} would be a more appropriate application rate). The T, NT, TD, and NTD treatments received an equivalent volume of water.

Figure 6.7: **Heavily trampled soil following grazing of a forage crop by dairy cows at Lincoln University's Ashley Dene farm (left); the author simulating animal trampling on a lysimeter (centre); typical appearance of soil following simulated trampling (right).**

6.2.3 Nitrous oxide emissions

A closed chamber method, similar to that of Hutchison & Mosier (1981), was used to determine N_2O emissions. A water filled trough on top of each lysimeter casing provided a gas-tight seal for the metal and polystyrene chamber used during sampling. At each sampling time, chambers were placed on top of the lysimeters for a total of 40 minutes, with three samples taken 20 minutes apart, i.e. t=0, t=20, and t=40 minutes. Samples were taken through a rubber septum located in the top of each chamber using a 60 ml syringe and hypodermic needle, and approximately 12 ml was placed in 6 ml glass vials. Samples for N_2O analysis were taken between 12:00 and 14:00 hours at each sampling event. Nitrous oxide concentration was analysed using gas chromatograph (SRI 8610GC, SRI Instrument, C.A., USA).

6.2.4 Soil mineral N

A sample of soil consisting of three cores, approximately 2.5 cm in diameter by 7.5 cm deep, was taken from each of the 32 companion soil blocks. Each of the 32 samples were thoroughly mixed, and five grams of soil was placed in another centrifuge tube before adding 25 ml of 2 M KCl. The tubes were then placed in a rack and shaken for 1 hour. After shaking, the solution was centrifuged for 10 minutes, before being filtered through Whatman #42 (or equivalent) filter paper. The filtrate was frozen until being analysed using a flow injection analyser (Tecator Inc. Sweden) for NH_4^+ and NO_3^-

concentration. The soil moisture content was calculated gravimetrically by drying approximately 10 g of soil at 105°C for a minimum of 24 hours.

6.2.5 Soil DCD concentration

In combination with each sampling event outlined in 6.2.3, five grams of soil from each of the TD, NTD, TUD80 and NTUD treatments were collected and placed in centrifuge tubes, before adding 25 ml of de-ionised water. The tubes were then placed in a rack and shaken for 1 hour. After shaking, the solution was centrifuged for 20 minutes, before being filtered through Whatman #42 (or equivalent) filter paper. The filtrate was frozen before being analysed using a High Performance Liquid Chromatograph (Shimadzu, Japan) for DCD concentration.

6.2.6 Plant growth and N uptake

In order to match practices at the Ashley Dene farm, on August 23rd, 2012, the soil was lightly cultivated and sown with oat seeds (*Avena sativa L.*) at a rate equivalent to 120 kg ha^{-1}, together with 15% potassic superphosphate at a rate equivalent to 100 kg ha^{-1}. The oats were harvested from the lysimeters on 28th November 2012, and from the soil rings on 4th December 2012. The herbage was dried at 65°C, weighed, and the number of shoots counted. A representative sample from each replicate grown on the lysimeters was taken for N concentration analysis using an Elemental Analyser (Elementar Vario MAX CN). The oat crop sown on 23rd August 2012 was harvested from the lysimeters on 28th November 2012, and from the soil rings on 4th December 2012.

6.2.7 Denitrifying microbial gene abundance

Additional samples of soil for assessing denitrifying microbial NO_2^- reductase *nirS*, and N_2O reductase *nosZ,* gene abundance were taken at the same times as the samples for mineral N and DCD assays. Approximately five grams of soil were packed into 6 ml plastic vials and stored at -80°C until analysis, using the methods described by Di *et al.* (2014) (Table 6.2). Briefly, DNA was extracted from the soil using MoBio Powersoil™ DNA isolation kits (MoBio Laboratories, GeneWorks Pty Ltd, South Australia, Australia) in accordance with the manufacturer's instructions. Quant-iT™ dsDNA BR assay kits, a Qubit fluorometer (Life Technologies, Auckland, New Zealand), and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Montchanin, USA), were used to determine the concentration and quality of the extracted DNA. For real-time qPCR analysis, samples were prepared using a CAS-1200 Robotic liquid handling system (Corbett Life Science, BioStrategy, Auckland, New Zealand), and analysed using a Rotor-Gene™ 6000 analyser (Corbett Life Science, BioStrategy, Auckland, New Zealand).

Table 6.2: Primer pairs and PCR conditions used in real-time qPCR analysis (Di *et al.* 2014).

Target group	Primer name	Sequence (5'-3')	Length of amplicon (bp)	Primer final concentration (nM)	Thermal profile	Amplification efficiency (R^2>0.99) (%)	References
nirS	Cd3aF R3cd	5'-GTSAACGTSAAGGARACSGG-3' 5'-GASTTCGGRTGSGTCTTGA-3'	410	750	95 °C for 2 min - x 1 cycle; 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, 85 °C for 20 s - x 40 cycles;	93-95	(Michotey *et al.* 2000, Throback *et al.* 2004)
nosZ	NosZ-F NosZ1622R	5'-CGYTGTTCMTCGACAGCCAG-3' 5'-CGSACCTTSTTGCCSTYGCG-3'	424	750	95 °C for 2 min – x 1 cycle; 95 °C for 20 s, 58°C for 30 s, 72 °C for 30 s, 85 °C for 15 s - x 40 cycles;	94-99	(Kloos *et al.* 2001, Throback *et al.* 2004)

6.2.8 Physical measurements

Bulk Density

Prior to cultivation on 23rd August 2012, samples were taken to determine the bulk density of the soil from un-disturbed portions of ten soil rings set aside for destructive sampling, using a sand replacement method. Samples were taken from five trampled, and five non-trampled soil rings. First, the surface of the soil was made flat, and then approximately 500 ml soil was extracted and placed in a paper bag. A plastic bag was then placed inside the cavity created by the excavated soil, into which dry sand of a known bulk density was carefully poured until the sand filled the void in the soil. The soil was dried in an oven at 105°C for a minimum of 24 hours, and weighed. The bulk density of the soil was calculated using Equation 6.2:

$$\mathrm{P_B} = \frac{M_{\mathrm{soil}}}{\mathrm{V_{soil}}} \qquad (6.2)$$

where P_B is the soil bulk density (Mg/m^3), M_{soil} is the mass of soil, and V_{soil} is the volume of soil, where $V_{soil} = M_{sand}/P_{sand}$, where M_{sand} is the mass of sand, and P_{sand} is the density of the sand.

The fine earth fraction of the soil was separated from the stones by sieving the dried samples using a 2 mm mesh sieve. The stones were washed, dried, and weighed to determine their relative contribution to total soil mass. The volume of stones was calculated by placing stones in a known volume of water and observing the mass of water displaced by the stones (density of water assumed to be 1 g cm^{-3}).

Air permeability

The stony nature of the Balmoral soil did not allow extraction of intact cores to measure the air permeability of the soil (Figure 6.8). It was difficult to find a site that was adequately representative of the entire field using small cores, as the micro-topography created by the animal hooves were larger than the core itself. Furthermore, it was not possible to find sites in the field that were free of stones to enable the cores to be pushed into the soil. Consequently, an in-situ method similar to that described by Iversen *et al.* (2001) was adopted (Figure 6.9).

The air permeability of the soil was measured on ten un-disturbed soil rings, giving five trampled, and five non-trampled replicates. A rubber channel was sealed to the top of each soil ring, to which a steel lid was clamped to provide an air tight seal. To create a flow of air through the soil, a cylinder of dry compressed air was connected via a regulator, to a variable area flow meter (0-60 L min^{-1} capacity), which was connected to the steel lid (Figure 6.9, Figure 6.10). A tube from the metal lid connected to a universal pressure module (GE Druck, Leicester, UK), which communicated to a differential pressure transducer (P26, Halstrup Walcher, Germany) with a range of 0-250 Pa.

Figure 6.8: Attempting to obtain cores for physical measurements of the soil. Note the rough surface and size of cow hoof prints relative to the corer.

To obtain measurements for the air permeability, the regulator on the gas cylinder was manipulated until steady flows of 5, 10, 20, or 25 L min^{-1} through the soil ring were reached. The pressure module was zero-calibrated to the ambient air pressure, and pressure readings taken at each of the four flow rates. The air permeability of the soil was calculated using Equation 6.3 and 6.4 (Liang *et al.* 1995):

(6.3)

$$k_a = \frac{\eta Q}{\Delta P A}$$

where *ka* is the air permeability (m^2), η is the gas viscosity (assumed to be 1.85 x 10^{-5} P), *Q* is the volumetric flow rate (m^3 s^{-1}), ΔP is the change in pressure between both sides of the soil (Pa), and *A* is a shape factor (m) (Equation 6.4):

(6.4)

$$A = 0.4862\left(\frac{D}{H}\right) - 0.0287\left(\frac{D}{H}\right)^2 + 0.1106$$

where *A* is the shape factor, *D* is the diameter of the soil ring, and *H* is the height of the soil within the soil ring.

Figure 6.9: Measuring the air permeability of the soil.

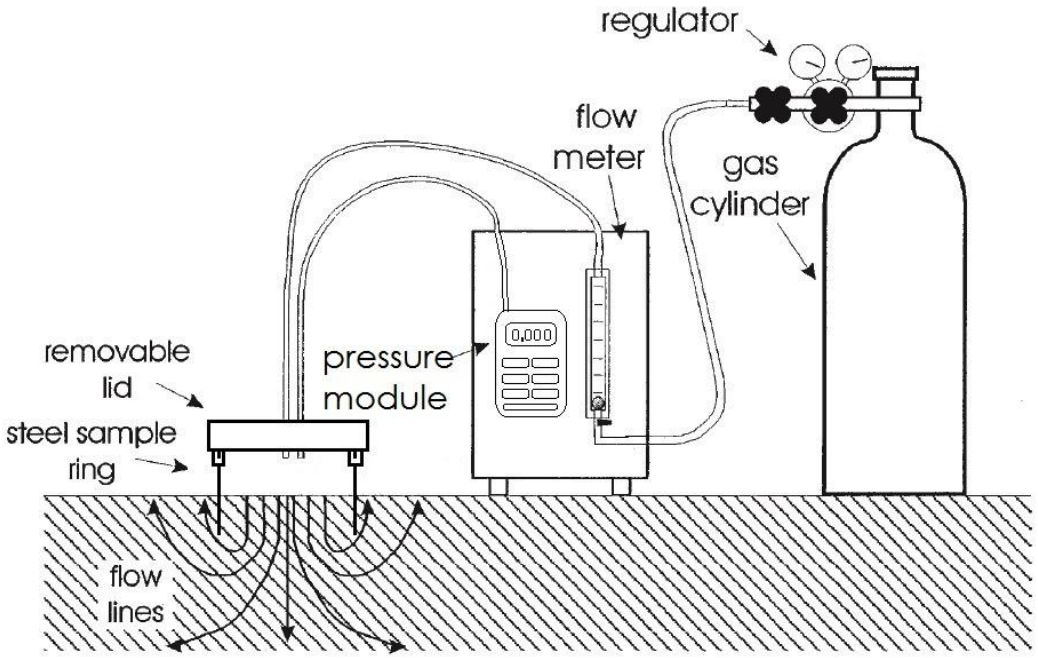

Figure 6.10: Apparatus for measuring the air permeability of soil (modified from Iversen *et al.* (2001)).

Soil moisture

To determine the effect of animal trampling on soil moisture in the lysimeters, tensiometers were installed in four trampled, and four non-trampled lysimeters. The tensiometers were constructed in accordance with Figure 6.11. The tensiometers were installed in the lysimeters by drilling a 10 mm hole which penetrated 10 cm into the side of the steel casing 5 cm below the soil surface of each of the eight lysimeters. A tensiometer was inserted into the hole in each of the eight lysimeter and pushed into the soil to ensure contact with the porous cup. The tensiometer was sealed to the lysimeter casing using silicon. Reflective foil was used to shield the sensors and lysimeter walls from heating by the sun (Figure 6.12).

Information was transferred from the sensors to a Campbell Scientific CR23X data logger, and telemetered via a Campbell Scientific CR411 radio. The radio signal was sent to the Lincoln University Intranet, via Campbell Scientific CR411 radio and NL100 network link interface. An air temperature sensor (Campbell Scientific, CS107) was installed into the side of one lysimeter, and a soil temperature sensor (Campbell Scientific, CS107) installed at 5 cm depth into another lysimeter, which were linked to the data logger. The logger applied calibration equations that changed the raw signal data into real values, and also compensated for temperature variation. Information was collected every ten minutes. The tensiometers were operational on the 20th July, 2012, and data was reported as kPa, and collected until October 2012.

Before the initial operation, and again at a maximum interval of two weeks, the tensiometers were primed by, removing the rubber bung in the end of the tube, and filling the tubes with de-gassed water using a 60 ml syringe and plastic tube. The de-gassed water had been boiled at room temperature under vacuum for about 10 minutes to remove dissolved gases, to reduce the chance of air bubbles forming in the tensiometer from dissolved air coming out of solution.

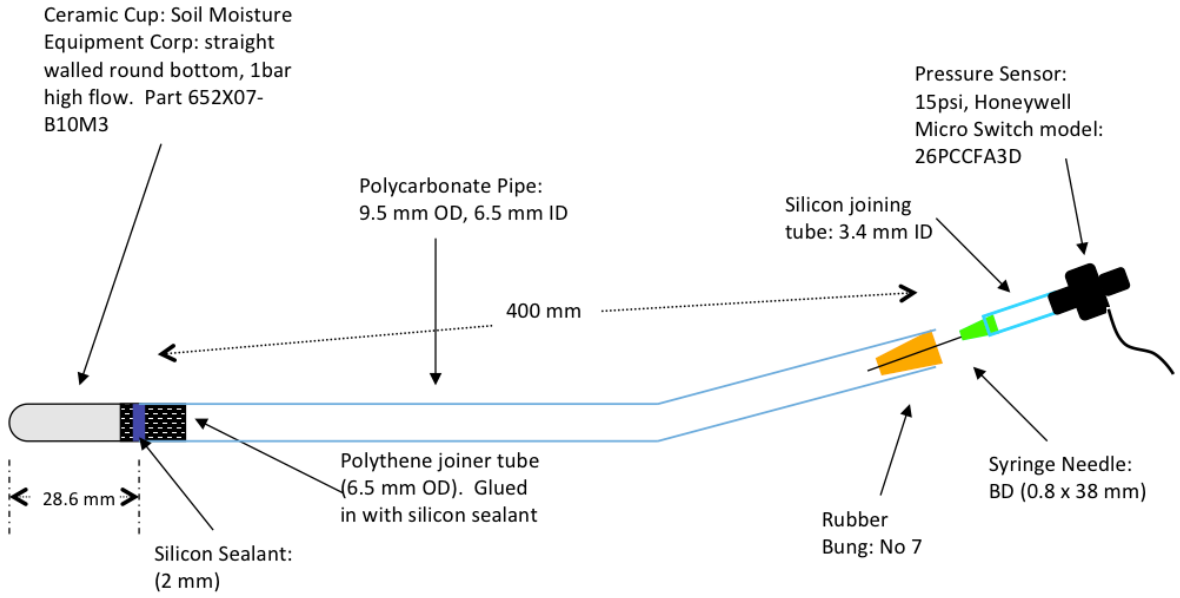

Figure 6.11: Schematic representation of a tensiometer inserted into lysimeters (courtesy Neil Smith).

Figure 6.12: Tensiometer installed in a lysimeter. The polycarbonate tube is visible exiting the lysimeter, with a pink rubber bung and sensor attached. Reflective foil was used to prevent heating of the lysimeter wall and sensor by the sun.

Water filled pore space

Water filled pore space (WFPS) was calculated using Equation 6.5 (Linn and Doran 1984):

$$WFPS = \frac{\theta_v}{TP} \quad (6.5)$$

where θ_v is the percent volumetric water content (%θ_m)(P_B), and TP is the percent total soil porosity (1 - P_B/P_P) (100), where P_P is the soil particle density (assumed to be 2.65 g cm^{-3}), P_B is the soil bulk density (g cm^{-3}), and θ_m is the gravimetric water content (g g^{-1}).

6.2.9 Data analysis

Nitrous oxide emission rates were calculated linearly from the increase in concentration of N_2O between the first and third gas samples taken at each sampling event. Daily N_2O fluxes were then calculated on the assumption that the calculated hourly flux represented the average hourly flux for that day. Total N_2O emissions were calculated by integrating the daily emission fluxes.

The emission factor (EF_3), or proportion of applied N that was emitted as N_2O was calculated using Equation 6.6 (de Klein *et al.* 2003):

$$EF(\%) = \frac{N_2O\text{-}N\ total\ (treatment) - N_2O\text{-}N\ total\ (control)}{UrineN\ (applied)}\ x\ 100 \quad (6.6)$$

where *EF(%)* is the emission factor, *N_2O-N total (treatment)* is the cumulative total N_2O emitted from a urine treatment, *N_2O-N total (control)* is the cumulative total N_2O emitted from the comparative no-urine treatment, and *UrineN (applied)* is the amount of N added as urine.

The average concentrations of NH_4^+, NO_3^-, and abundance of gene copies throughout the duration of the experiment were determined by taking the average of the daily concentration between sampling events.

Least significant differences and P-values were calculated following analysis of variance using the GenStat software package (version 16, VSN International Ltd, UK). Soil mineral nitrogen concentrations required a log transformation to determine differences between treatments.

6.3 Results

6.3.1 Climate

During the monitoring period, 290 mm of rain fell, which was supplemented with 213 mm of simulated rainfall, plus 107 mm of summer irrigation (Figure 6.13). The maximum daily rainfall was 40.4 mm on day 114. Water input to the soil blocks was matched to that of the lysimeters. The mean daily air temperature ranged from 2.1°C to 18.2°C (Figure 6.14). The lowest mean daily air temperature was 2.1°C on day 9, and the highest mean daily air temperature was 18.2°C on day 134.

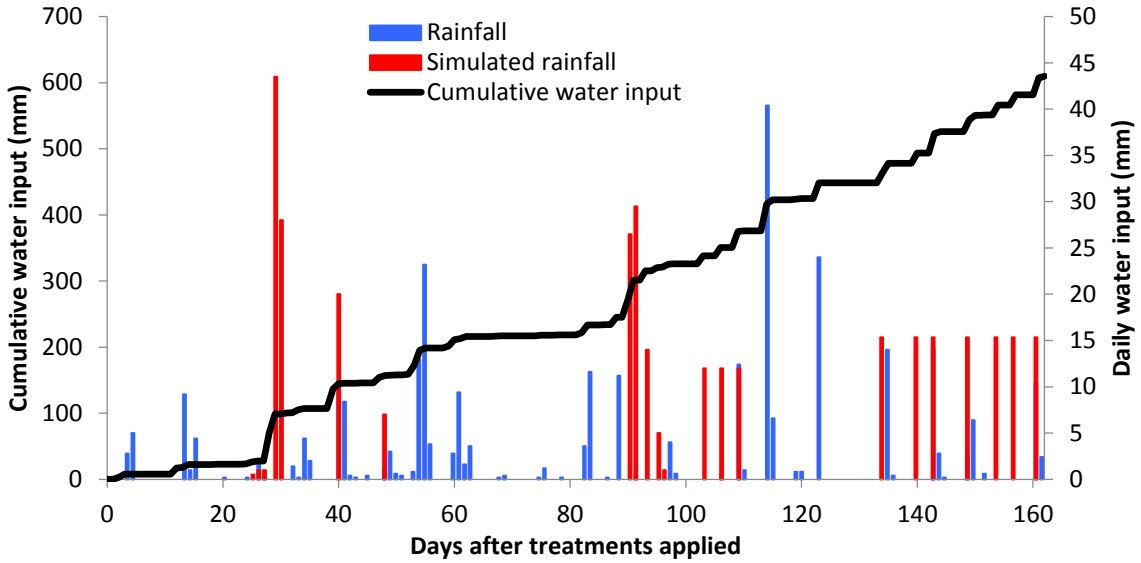

Figure 6.13: Cumulative and daily water input to the lysimeters, from 21st July to 30th November 2012.

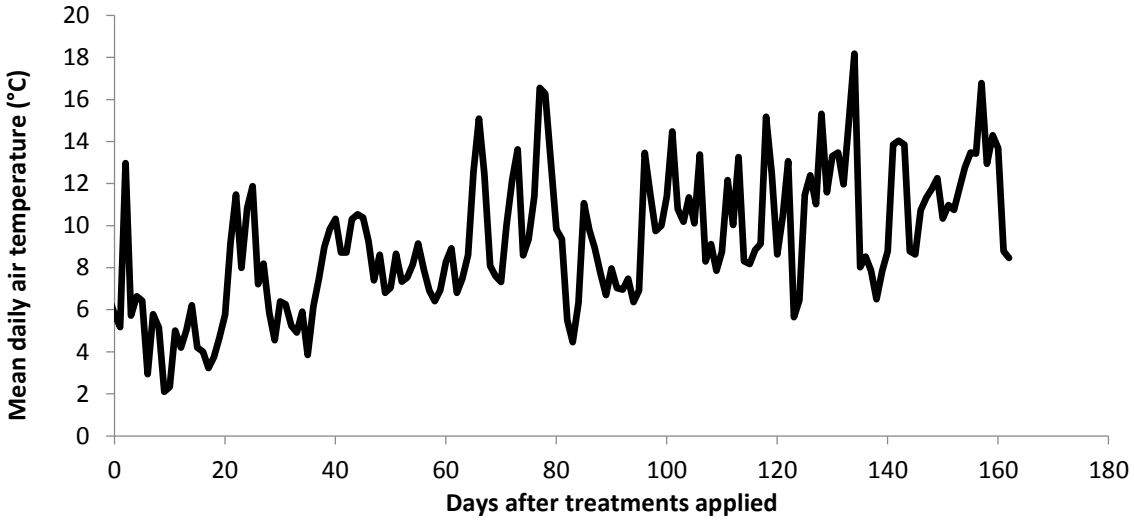

Figure 6.14: Mean daily air temperature at the experimental site during the monitoring period, from 21st July to 30th November 2012.

6.3.2 Nitrous oxide emissions

Peak N_2O emission flux

The three highest N_2O emission flux peaks were observed from the trampled soil with urine (TU). The highest peak was observed 110 days after treatments were applied when 180 g N_2O-N ha^{-1} day^{-1} was emitted, the second highest was observed after 47 days when 174 g N_2O-N ha^{-1} day^{-1} was emitted, and the third highest peak observed after 89 days when 167 g N_2O-N ha^{-1} day^{-1} was emitted (Figure 6.15a). The three peaks were not significantly different. A peak N_2O flux from the non-trampled soil with urine (NTU) was observed after 47 days, when 154 g N_2O-N ha^{-1} day^{-1} was emitted (Figure 6.15a).

When DCD was sprayed on the soil prior to trampling and cow urine application, the peak N_2O emission flux was observed 103 days after treatments were applied, when the trampled soil with urine and DCD (TUD) emitted a peak of 147 g N_2O-N ha^{-1} day^{-1} (Figure 6.15b). The peak N_2O flux from the non-trampled soil with urine and DCD (NTUD) occurred earlier, after 47 days, when 85 g N_2O-N ha^{-1} day^{-1} was emitted (Figure 6.15b). The peak flux from the NTUD soil was significantly less than from the TUD, NTU, and TU soil, however the peak flux from the TUD was not significantly different than that from the NTUD.

The peak emission flux from the trampled soil with no urine or DCD (T) was 28 g N_2O-N ha^{-1} day^{-1}, which occurred 41 days after treatments were applied, and on the same day, peak emissions were observed from the trampled soil with DCD (TD), when 29 g N_2O-N ha^{-1} day^{-1} was emitted, and the non-trampled soil (NT), where 14 g N_2O-N ha^{-1} $day^{-1}$was emitted (Figure 6.15a, b). The peak N_2O flux from the non-trampled soil with DCD (NTD) was 45 g N_2O-N ha^{-1} day^{-1}, which occurred after 32 days (Figure 6.15b).

Total N_2O emissions

Over the 159 day measurement period, the trampled soil with urine (TU) released 7.20 kg N_2O-N ha^{-1}, which was significantly more than all other treatments (Table 6.3, Figure 6.16a). The non-trampled soil with urine (NTU) released 3.48 kg N_2O-N ha^{-1}, which was significantly more than the non-trampled soil without urine (NT), which emitted 0.75 kg N_2O-N ha^{-1}. The trampled soil without urine (T) emitted 1.11 kg N_2O-N ha^{-1}, which was not significantly different to that from the NT soil, and significantly less than the TU soil ($P<0.05$). EF_3 values were 1.5% from the TU treatment, and 0.9% from the NTU treatment. Applying DCD to the soil reduced total N_2O emissions from the TU soil by 44% to 4.06 kg N_2O-N ha^{-1} (TUD) ($P<0.05$). However, applying DCD to non-trampled soil had no statistically significant effect on N_2O emissions (Figure 6.16a, b). The EF_3 values were not significantly different between DCD treatments at 0.7% from the TUD, and 0.9% from the NTUD treatments.

Table 6.3: Total cumulative N_2O emissions, reduction in N_2O emissions following DCD application, and proportion of applied N emitted as N_2O from lysimeters during the period 21st June to 27th November 2012.

Treatment	Total emissions (kg N_2O-N ha^{-1})	% Reduction	EF_3 (%)
T	1.11	-	-
TD	0.91	-	-
TU	7.20	-	1.5
TUD	4.06	44	0.7
NT	0.75	-	-
NTD	1.45	-	-
NTU	3.48	-	0.7
NTUD	4.19	-	0.9
LSD# (0.05)	2.17	-	-

#Least significant difference ($P < 0.05$).

6.3.3 Soil mineral N and DCD concentrations

Ammonium

All soil NH_4^+ concentrations of all the urine amended treatments (TU, NTU, TUD, and NTUD) seven days after urine application were greater than 139 mg NH_4-N kg $soil^{-1}$, and were not significantly different between treatments (Figure 6.17a, b). After 50 days, the concentration of NH_4^+ within the soil of the TU and NTU treatments had reached background levels. Applying DCD to the soil following urine application increased the time taken for the concentration of NH_4^+ within the soil of the NTUD treatment to reach background levels by 13 days when compared to the NTU soil. Meanwhile, the TUD treatment took 126 days before the NH_4^+ concentration reached background levels (Figure 6.17b).

The average concentration of NH_4^+ throughout the duration of the experiment was highest in the trampled soil with urine and DCD (TUD) ($P<0.05$), followed by the non-trampled soil with urine and DCD (NTUD), the trampled soil with urine (TU), and the non-trampled soil with urine (NTU) (Table 6.4). The average concentration of NH_4^+ was not different between the TU, NTU, and NTUD soils. Of the treatments that did not receive urine, the trampled soil with DCD (TD) had the highest average NH_4^+ concentration, followed by the non-trampled soil with DCD (NTD), the trampled soil without urine or DCD (T), and finally, the lowest average NH_4^+ concentration was found in the non-trampled soil without amendments (NT) (Table 6.4).

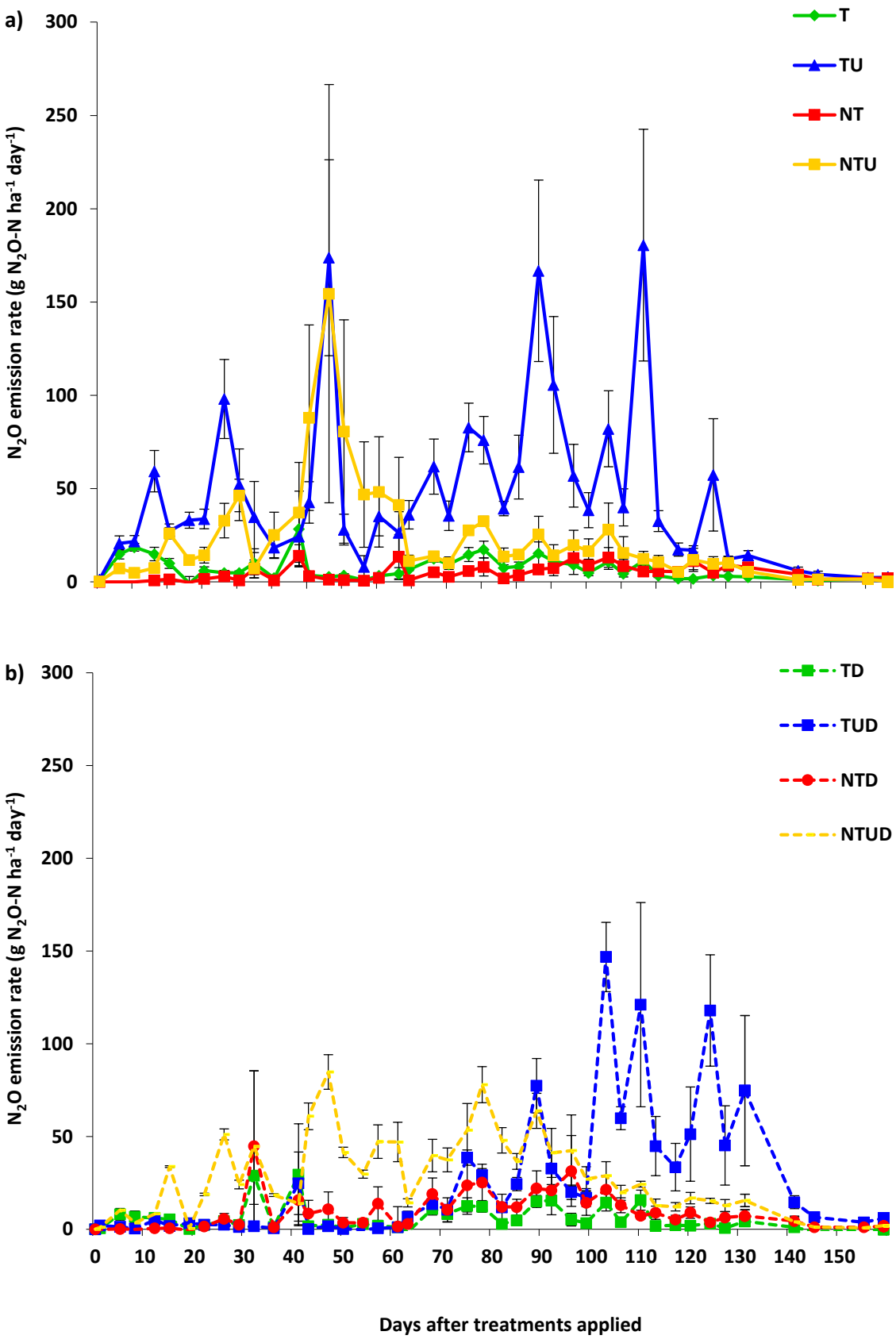

Figure 6.15: **Daily N_2O emission flux from a) treatments where no DCD was applied, and b) treatments where DCD was applied. Error bars represent on standard error of the mean.**

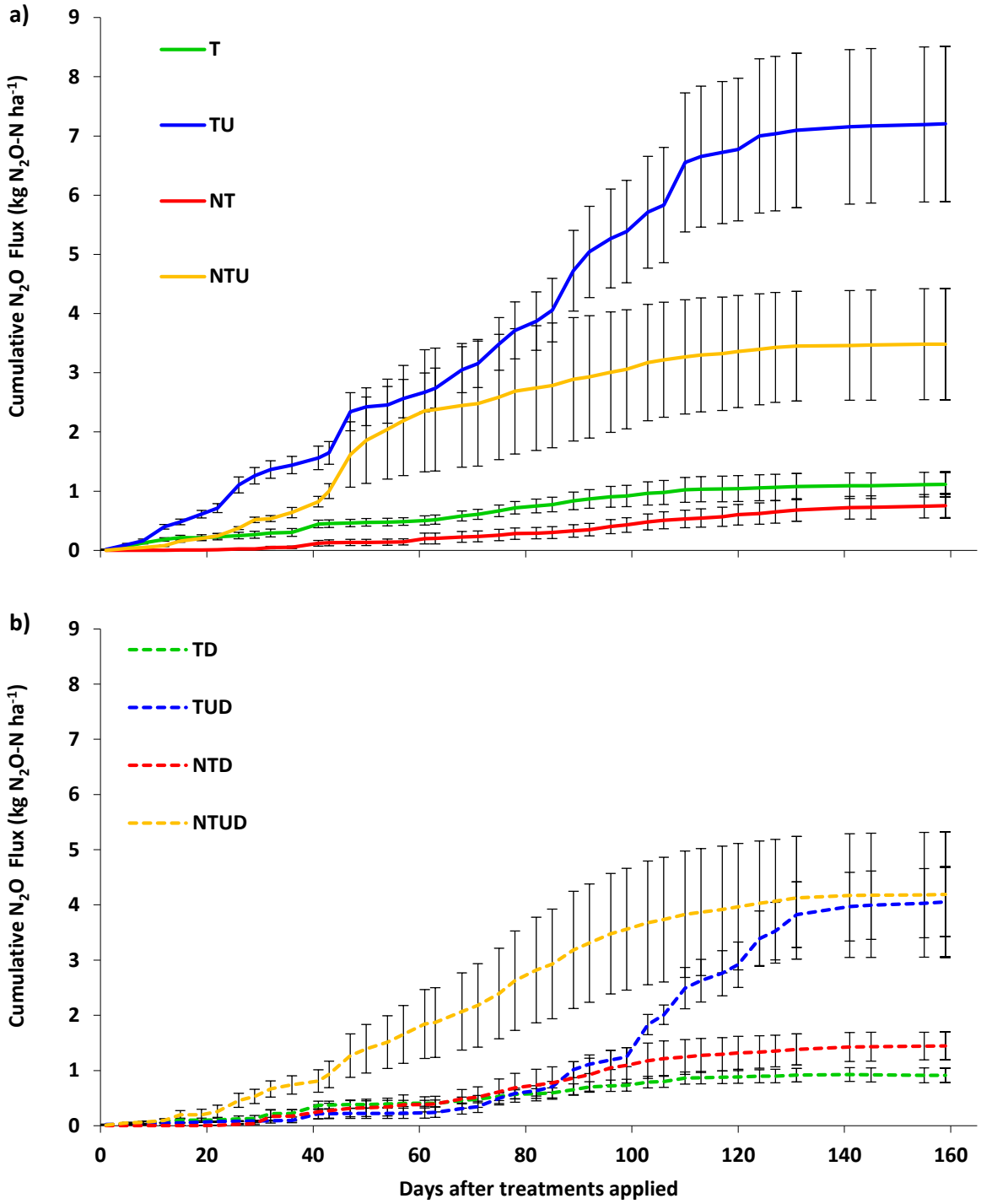

Figure 6.16: **Cumulative N_2O emission from a) lysimeters without DCD, and b) lysimeters with DCD. Error bars represent one standard error of the mean.**

Nitrate

The peak NO_3^- concentration in the non-trampled soil with urine (NTU) was 48.8 mg NO_3-N kg $soil^{-1}$ (Figure 6.18a), which occurred 37 days after treatments were applied. The peak NO_3^- concentration in the trampled soil with urine (TU) was not significantly different to the NTU at 44.2 mg NO_3-N kg $soil^{-1}$, but it occurred 50 days after treatments were applied (Figure 6.18a).

The highest NO_3^- concentration of 59.7 mg NO_3-N kg $soil^{-1}$ was observed in the trampled soil with urine and DCD (TUD), 105 days after treatments were applied, however this peak was not significantly different from the peaks observed for the NTU, or TU soil (Figure 6.18a, b). The lowest peak NO_3^- concentration ($P<0.05$) in treatments where urine was applied was 19.7 mg NO_3-N kg $soil^{-1}$, and was seen in the non-trampled soil with urine and DCD (NTUD) 63 days after treatments were applied (Figure 6.18b).

The average concentration of NO_3^- throughout the duration of the experiment was highest in the trampled soil with urine (TU), followed by the non-trampled soil with urine (NTU), the trampled soil with urine and DCD (TUD), and the non-trampled soil with urine and DCD (NTUD) (Table 6.4). Of the treatments that did not receive urine, the trampled soil without urine or DCD (T) had the highest average NO_3^- concentration, followed by the non-trampled soil with DCD (NTD), the trampled soil without urine or DCD (T), and finally the lowest average NO_3^- concentration was found in in the non-trampled soil without amendments (Table 6.4).

Table 6.4: Average mineral nitrogen and DCD concentration in the soil over the duration of the experiment, from 21st June to 25th October 2012.

Treatment	Average NH_4^+ (mg NH_4-N kg $soil^{-1}$)		Average NO_3^- (mg NO_3-N kg $soil^{-1}$)		Average DCD (mg DCD kg $soil^{-1}$)
	Log_{10} NH_4-N	BT mean	Log_{10} NO_3-N	BT mean	
T	0.42	2.7	0.37	2.4	-
TD	0.81	6.6	0.12	1.3	1.8
TU	1.42	26.2	0.99	9.9	-
TUD	1.67	47.9	0.63	4.5	1.9
NT	0.27	1.9	0.23	1.7	-
NTD	0.47	2.9	0.20	1.6	0.6
NTU	1.21	17.7	0.91	8.2	-
NTUD	1.50	32.9	0.60	4.1	0.7
LSD# (0.05)	0.18	-	0.15	-	0.9

#Least significant difference ($P < 0.05$).

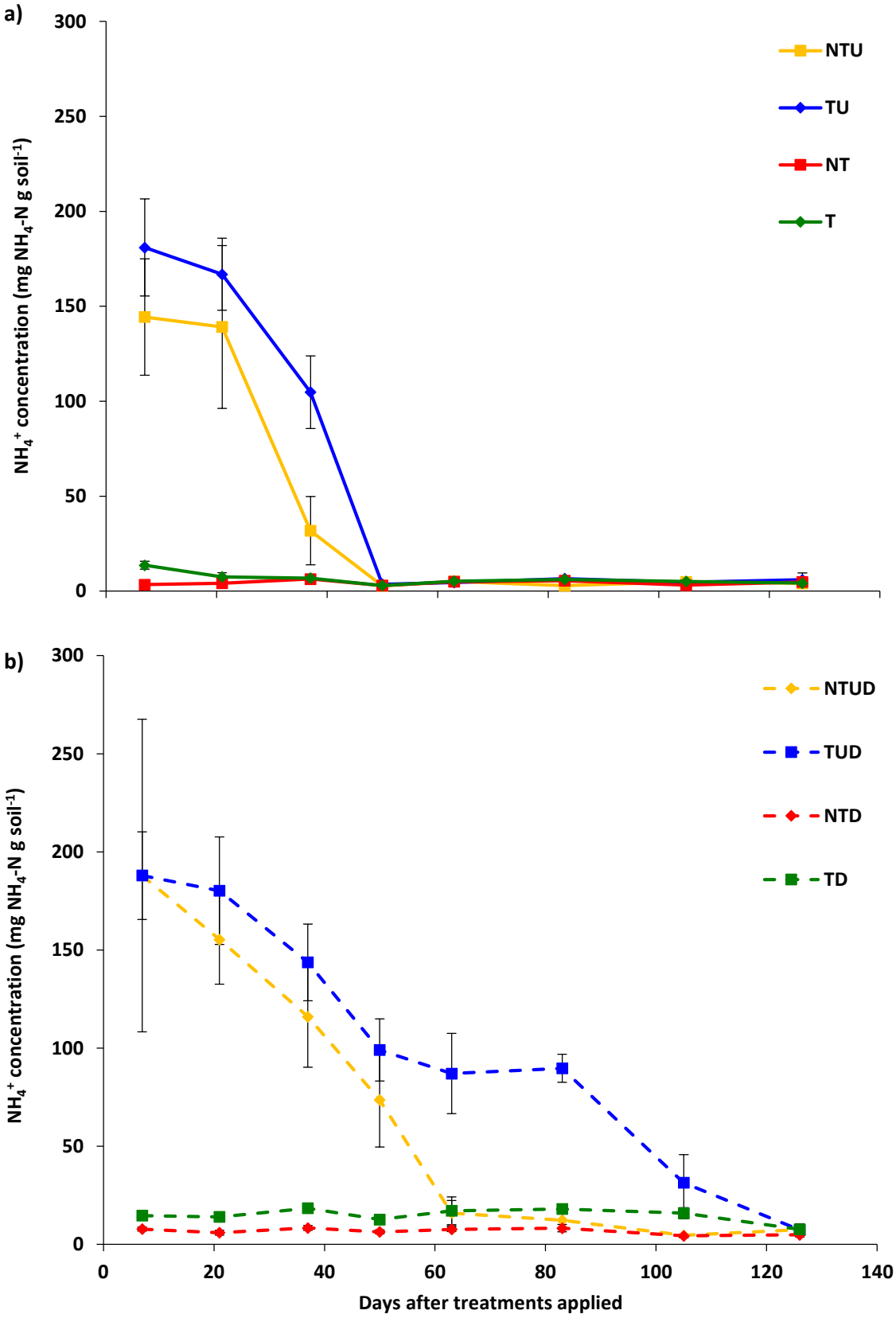

Figure 6.17: **Ammonium concentration in the soil of the urine affected treatments a) without DCD and b) with DCD, during the period from 21st June to 25th October 2012. Error bars represent one standard error of the mean.**

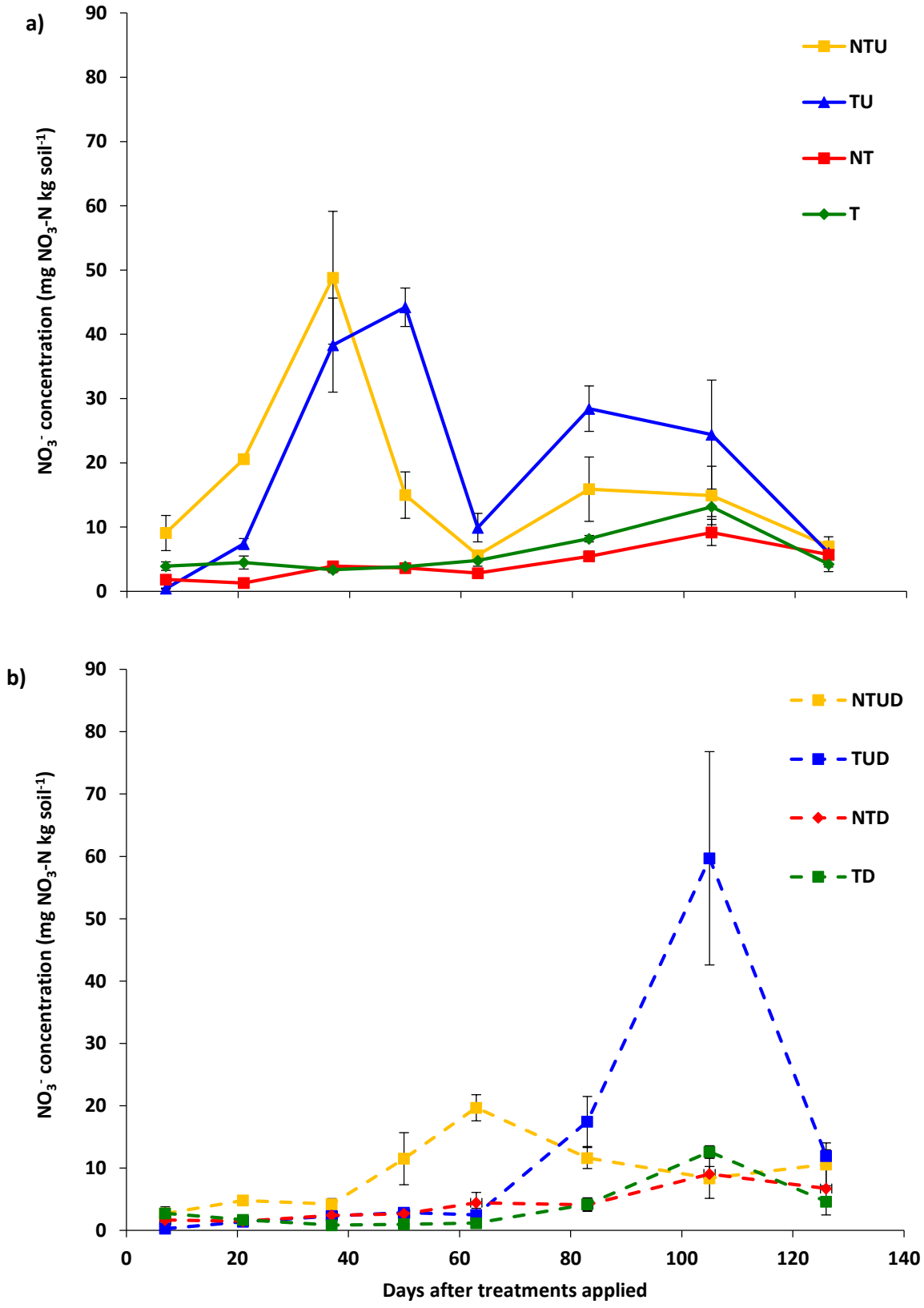

Figure 6.18: **Nitrate concentration in the soil of the treatments a) without DCD and b) with DCD, during the period from 21st June to 25th October 2012. Error bars represent one standard error of the mean.**

DCD concentration

The concentration of DCD in the non-trampled soil was not detectable 63 days after application, whereas the concentration of DCD in the trampled soil was not detectable 126 days after application. Consequently, the concentration of DCD in the soil was, on average, higher ($P<0.05$) in both trampled treatments than in both non-trampled treatments (Figure 6.19, Table 6.4).

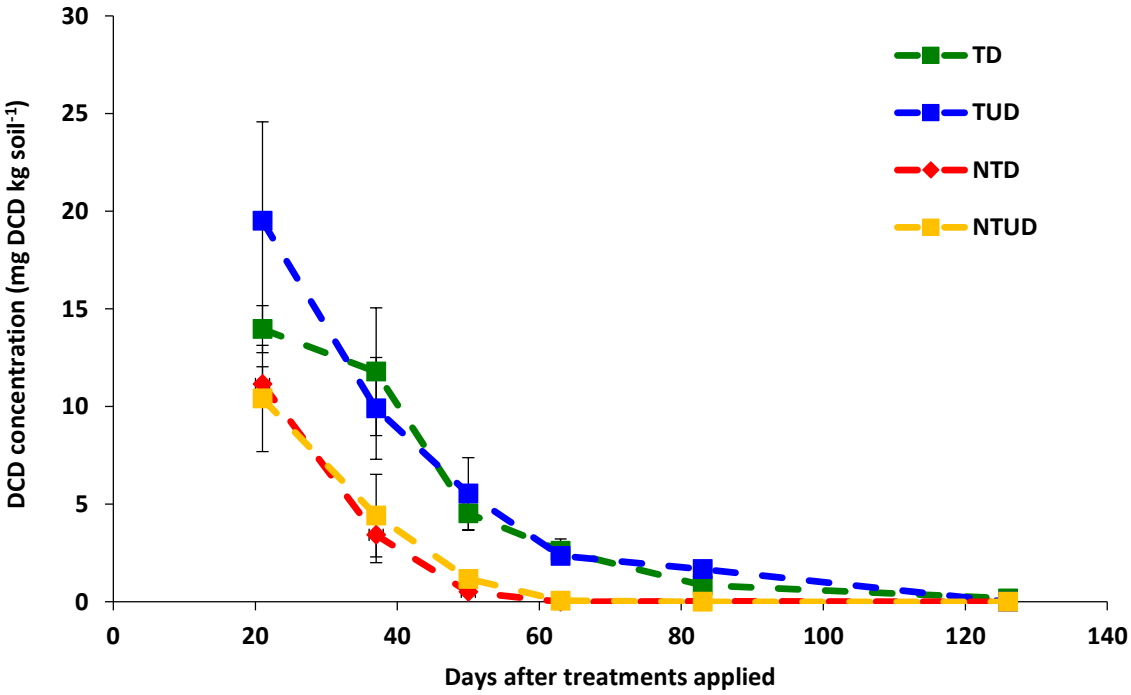

Figure 6.19: **Concentration of DCD in the soil during the experimental period. Error bars represent one standard error of the mean.**

6.3.4 Plant growth

The trampled soil with urine and DCD (TUD) grew more dry matter (DM) per plant than all other treatments (Figure 6.20, Table 6.5) ($P<0.05$). The mean nitrogen concentration of the oats harvested from the lysimeters ranged from 1.11% to 1.39% (Table 6.5), and the combination of urine and DCD in both trampled and non-trampled soil produced the highest plant N concentration, however differences between all treatments were small. The amount of plant matter grown in the TUD soil was more than twice that of any other treatment ($P<0.05$), while there was no significant difference in the amount of plant matter grown in all other treatments (Figure 6.20, Figure 6.21). The amount of plant matter grown, and the higher plant N concentration, meant the plants grown in the TUD soil removed 56.8 kg N ha^{-1}, which was more than any other treatment ($P<0.05$).

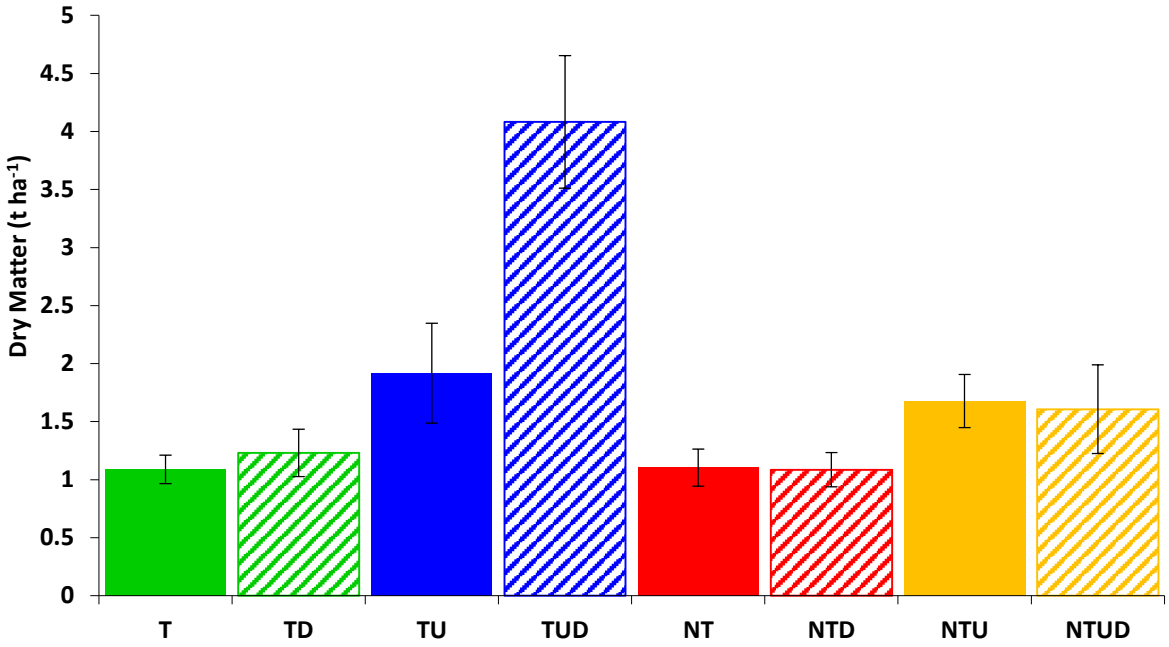

Figure 6.20: **Amount of dry matter grown in the lysimeters and soil rings between 23rd August and 28th November (lysimeters) and 4th December (soil rings). Error bars represent one standard error of the mean.**

Figure 6.21: **Vigorous growth of oat crop in trampled soil with urine and DCD (left), compared with growth in non-trampled soil with urine (centre), and trampled soil with urine (right). Photograph taken 13th November, 2012.**

Table 6.5: Average number of plants per plot, average dry matter, and N removal by an oat crop grown in lysimeters and soil rings between 23rd August 2012, and the conclusion of the experiment.

Treatment	Number of plants	DM (kg ha^{-1})	Plant N concentration## (%)	N removal### (kg ha^{-1})
T	23	1,089	1.3	13.7
TD	20	1,231	1.1	13.7
TU	25	1,917	1.1	21.4
TUD	26	4,083	1.4	56.8
NT	17	1,105	1.3	14.3
NTD	19	1,086	1.2	12.9
NTU	17	1,485	1.2	19.9
NTUD	19	1,607	1.4	21.9
LSD# (0.05)	5	903	0.2	-

#Least significant difference (P < 0.05). ## Mean N concentration of plants collected from lysimeters. ### N removal calculated on mean DM collected from lysimeters and soil rings, and mean N concentration of plants collected from lysimeters.

6.3.5 Denitrifying microbial gene abundance

Throughout the duration of the experiment, the abundance of NO_2^- reducing *nirS* gene copies were, on average, lower in the trampled soil with urine (TU) when compared with all other treatments (P<0.05) (Figure 6.22a,Table 6.6). The average abundance of *nirS* gene copies were not significantly different between all other treatments. The peak *nirS* gene copy numbers for all treatments were observed 37 days after treatments were applied (Figure 6.22a, b).

The abundance of N_2O reducing *nosZ* gene copies were, on average, lower in both trampled and non-trampled soil when urine was applied (TU and NTU) (Figure 6.23a,Table 6.6). However, there was no statistical difference in average *nosZ* gene copy numbers between the other treatments (Figure 6.23a,b, Table 6.6).

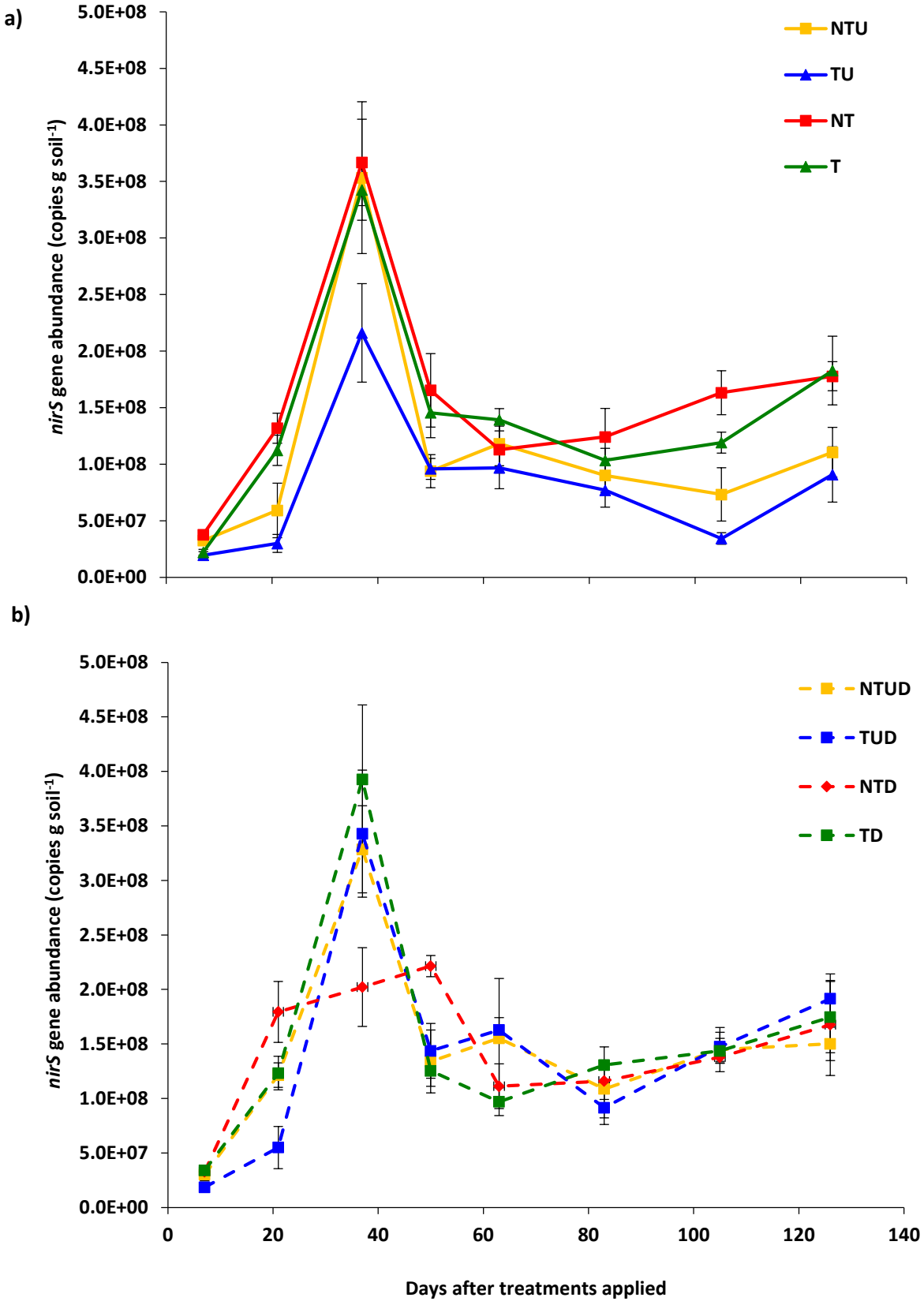

Figure 6.22: **Denitrifying NO_2^- reductase *nirS* gene copy number in a) the soil without DCD, and b) the soil with DCD, during the period from 21st June to 25th October 2012. Error bars represent one standard error of the mean.**

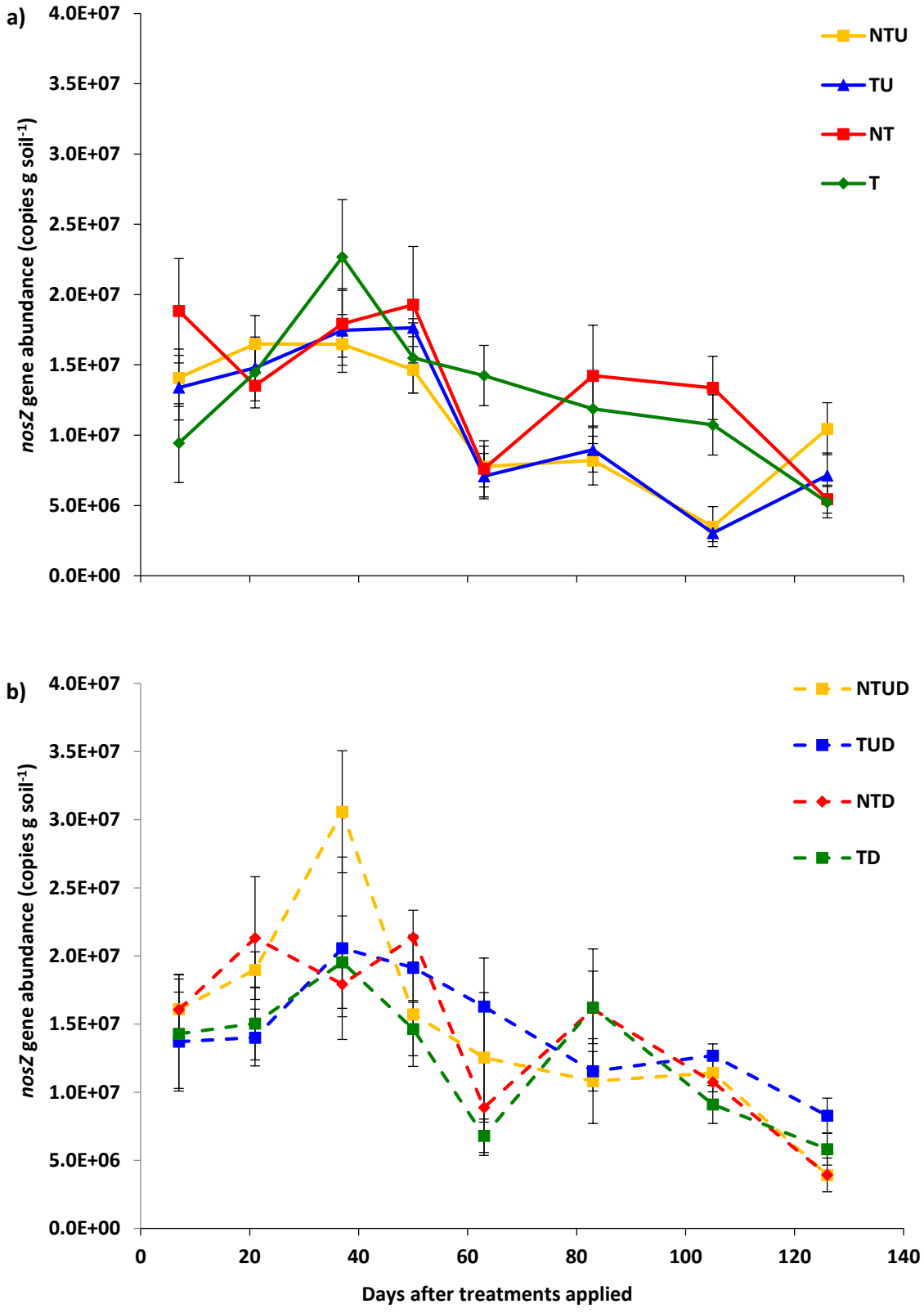

Figure 6.23: **Denitrifying N_2O reductase *nosZ* gene copy number in a) the soil without DCD, and b) the soil with DCD, during the period from 21st June to 25th October 2012. Error bars represent one standard error of the mean.**

Table 6.6: Average abundance of microbial denitrifying NO_2^- reductase *nirS*, and N_2O reductase *nosZ* gene copies in the soil over the duration of the experiment, from 21st June to 25th October 2012.

Treatment	Average *nirS* abundance (gene copies g soil^{-1})	Average *nosZ* abundance (gene copies g soil^{-1})
T	1.5 x 10^8	1.3 x 10^7
TD	1.6 x 10^8	1.3 x 10^7
TU	8.5 x 10^7	1.1 x 10^7
TUD	1.5 x 10^8	1.4 x 10^7
NT	1.5 x 10^8	1.4 x 10^7
NTD	1.4 x 10^8	1.5 x 10^7
NTU	1.2 x 10^8	1.0 x 10^7
NTUD	1.5 x 10^8	1.5 x 10^7
LSD# (0.05)	3.1 x 10^7	3.1 x 10^6

#Least significant difference (P < 0.05).

6.3.6 Physical measurements

Soil bulk density

The bulk density of the fine earth fraction of the trampled soil was 1.11 g cm^{-3}, while the bulk density of the fine earth fraction of the non-trampled soil was significantly lower (P<0.05) 0.89 g cm^{-3} (Table 6.7). The soil had, on average, 47% stones by volume. There was no significant difference in the whole-soil bulk density values between trampled and non-trampled soil (which is dominated by the high stone content).

Table 6.7: Bulk density of whole soil, and bulk density of fine earth fraction, with volume of stones greater than 2 mm. Samples taken 21st August, 2012 – 61 days after treatments were applied.

Treatment	Whole soil bulk density (g cm^{-3})	Fine earth bulk density (g cm^{-3})	Stone content (% v/v)
Trampled	1.54	1.11	48
Non-trampled	1.34	0.89	46
LSD# (0.05)	0.23	0.20	-

#Least significant difference (P < 0.05).

Air permeability

The non-trampled soil had a greater air permeability (AP) than the trampled soil prior to simulated ploughing, however the range of the pressure module selected for measuring AP did not adequately match the precision of air flow delivery using the equipment available, and so the difference between treatments was not statistically significant (Table 6.8). Consequently, on some occasions where the resistance to air flow was high (such as in the trampled soil) it was not possible to obtain pressure readings at the lowest air flow rates, as the values were above the range of measurement of the pressure transducer. Nevertheless, when sufficient readings were obtained, a linear increase in chamber pressure was observed as airflow was increased, indicating the methodology was sound (e.g. Figure 6.24). Following simulated ploughing, the permeability of the trampled soil increased, and there was no statistical difference in the AP between the trampled and non-trampled soil.

Table 6.8: **Log and back transformed air permeability (AP) of trampled and non-trampled soil before and after simulated ploughing for establishment of oat crop.**

Treatment	AP – pre ploughing (m^2)		AP – post ploughing (m^2)	
	Log10 AP	BT Mean	Log10 AP	BT Mean
Trampled	-12.39	$<4.4 \times 10^{-13}$##	-11.91	2.4×10^{-12}
Non-trampled	-11.77	1.8×10^{-12}	-11.56	2.8×10^{-12}
LSD# (0.05)	0.16	-	0.54	-

#Least significant difference (P < 0.05). ## Some values below scale of measurement.

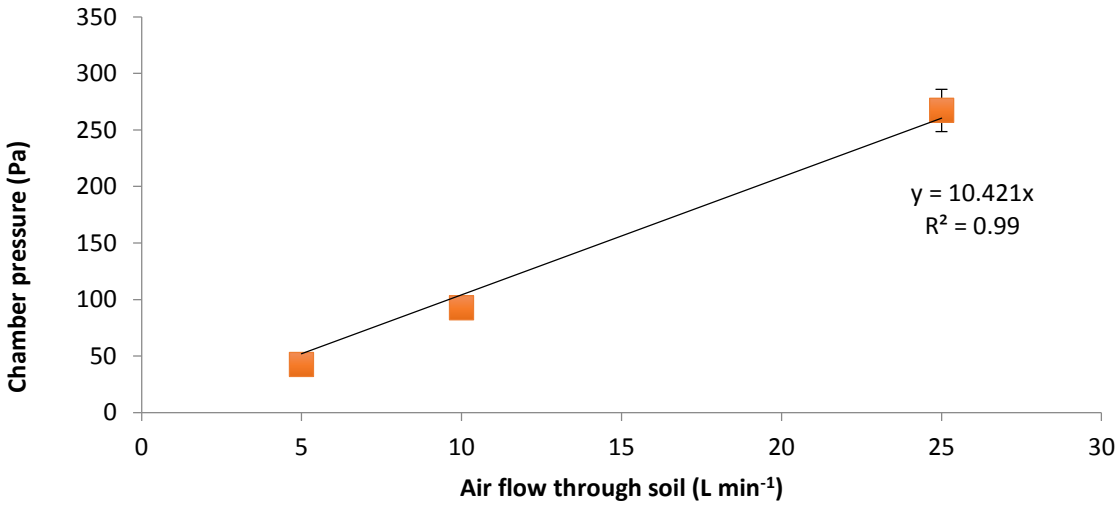

Figure 6.24: **Linear increase in chamber pressure relative to air flow through the soil as observed for non-trampled soil on 23rd August, 2012.**

Soil moisture

Two tensiometers (one trampled, and one non-trampled) failed to produce data, possibly because of the presence of stones, and were subsequently excluded from analysis. No statistically significant differences in the matric potential recorded by the remaining six tensiometers were observed between trampled and non-trampled soil (Figure 6.25). Likewise, the gravimetric soil moisture was very similar between the trampled and non-trampled soil when samples were taken for mineral N analysis. However, when considering the density of the treatments, the trampled soil had a higher volumetric water content (θ_v) than the non-trampled soil (Figure 6.26).

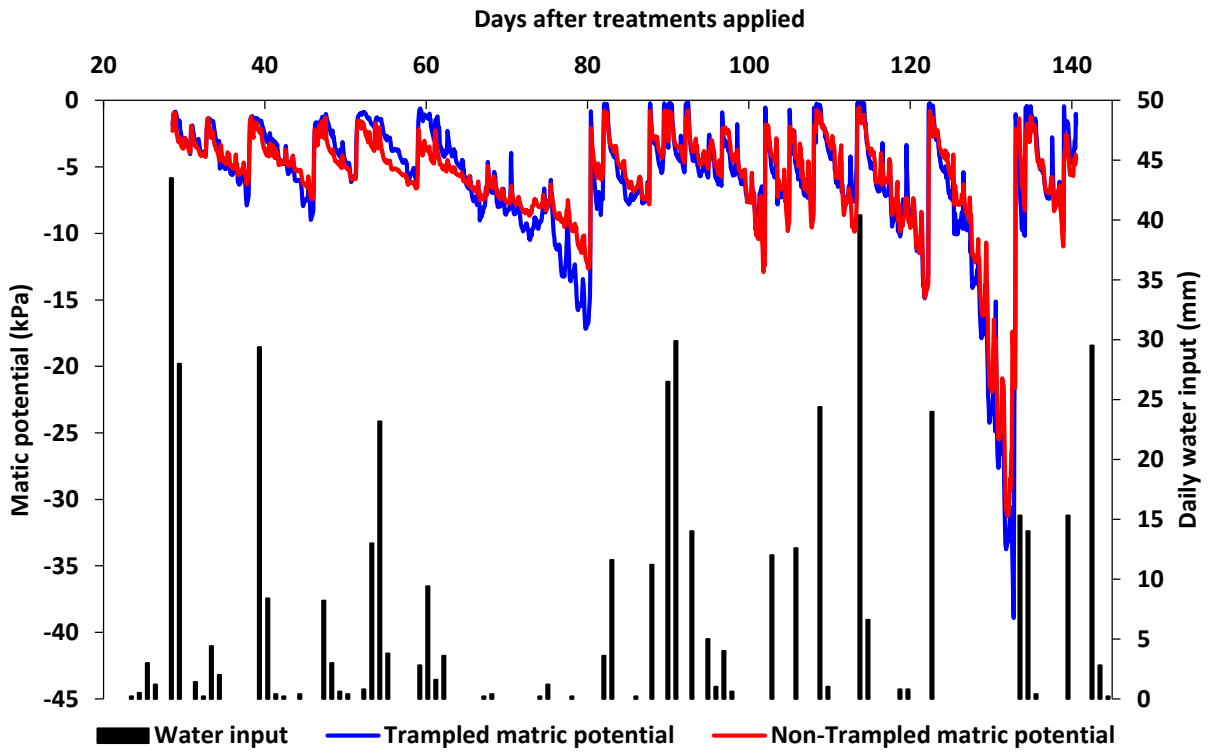

Figure 6.25: **Average matrix potential recorded by tensiometers in trampled and non-trampled lysimeters, with total daily water input.**

Water filled pore space

The water filled pore space was higher in the trampled soil than in the non-trampled soil at all measurement periods up until the soil was disturbed when the oats were sown ($P<0.05$). The WFPS of the trampled soil ranged from 40.9% to 47.9%, with an average of 44.6%, while the WFPS of the non-trampled soil ranged from 25.6% to 29.9%, with an average of 28.6% (Figure 6.27).

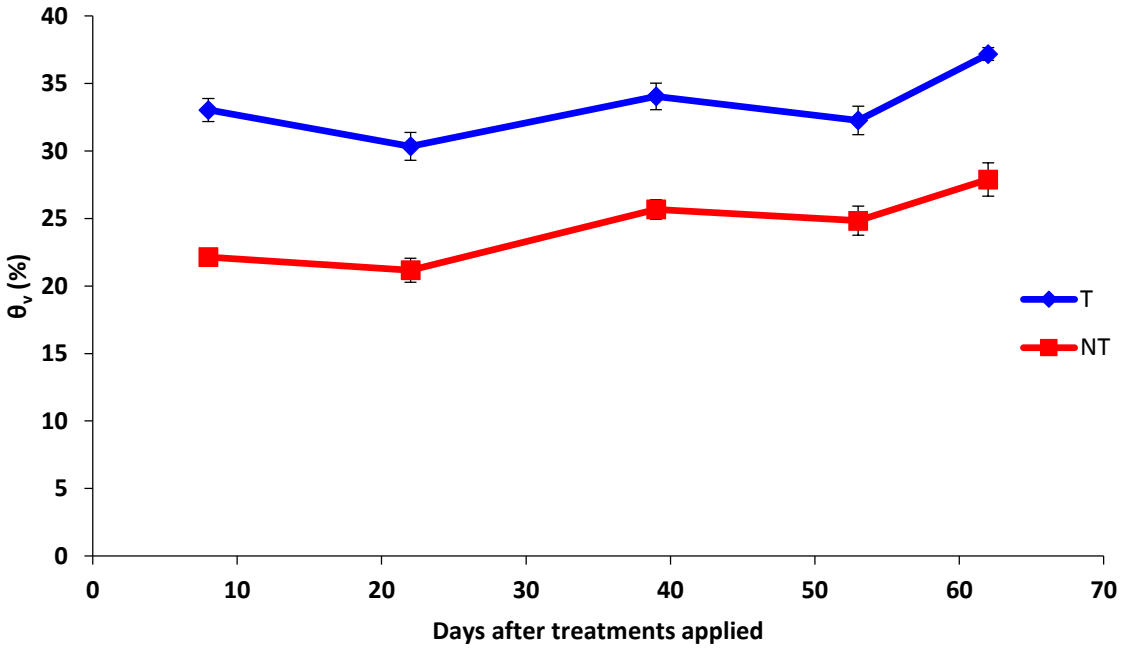

Figure 6.26: **Volumetric soil moisture content (θ_v) in trampled (T) and non-trampled (NT) soil in samples taken for mineral N analysis. Error bars represent one standard error of the mean.**

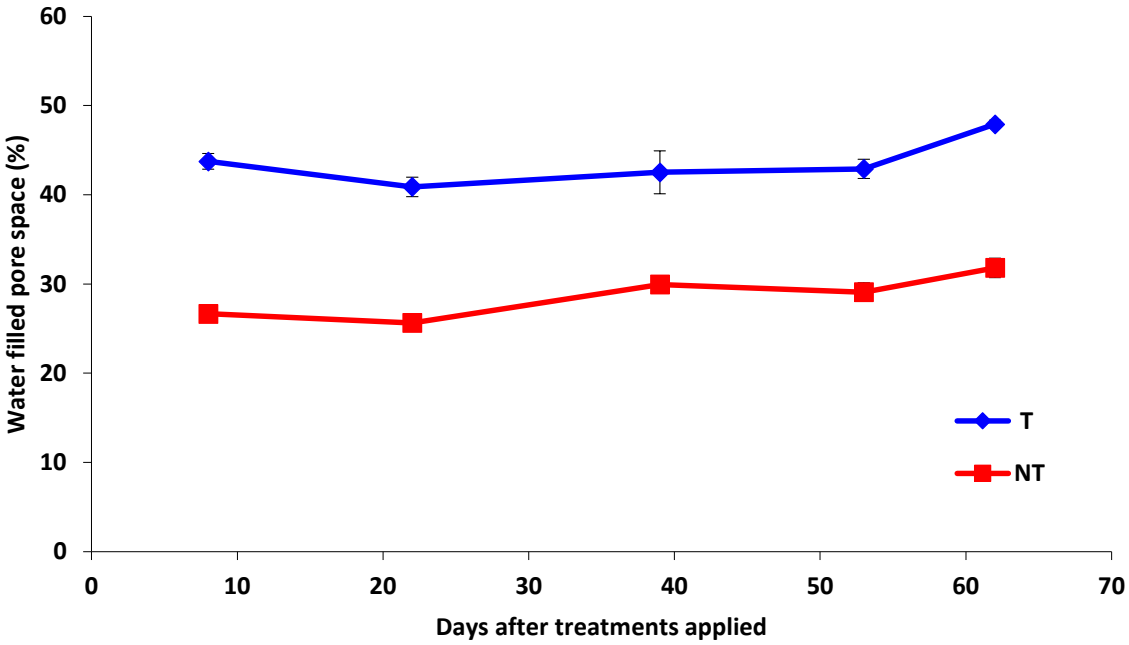

Figure 6.27: **Water filled pore space of trampled (T) and non-trampled (NT) soil. Error bars represent one standard error of the mean.**

6.4 Discussion

The trampled soil with dairy cow urine (TU) produced more N_2O than any other treatment during the 159 day monitoring period, emitting 7.20 kg N_2O-N ha^{-1} (Table 6.3). The non-trampled soil with urine (NTU) produced less than half as much N_2O as the TU soil, emitting 3.48 kg N_2O-N ha^{-1} (Table 6.3). Applying DCD to the soil which had received urine reduced total N_2O emissions by 44% ($P<0.05$) in the trampled soil (TUD), but did not significantly affect total N_2O emissions from the non-trampled soil (NTUD). Hill (2014) reported that more DCD was leached at a faster rate from non-trampled soil than from trampled soil, which agrees with the lower concentration of DCD extracted from the non-trampled soil (Table 6.4). The concentration of DCD in the non-trampled soil was almost un-detectable 50 days after it was applied (Figure 6.19), which could possibly explain why DCD was not effective in reducing N_2O emissions in this treatment. The peak N_2O emission fluxes were not significantly different between the TU, NTU, or TUD treatments, but the peak N_2O flux from the NTUD soil was lower than all other urine treatments. While the NTU, TUD, and NTUD treatments produced only one significant emission peak, the TU soil produced three peak fluxes of similar magnitude at different stages of the experiment (Figure 6.15a, b).

In the first field trial reported in this thesis (Chapter Four), urine was applied to trampled soil at a rate equivalent to 700 kg N ha^{-1}, and in the present experiment, urine was applied at the rate of 400 kg N ha^{-1}. Despite the difference in N application rate, the trampled soil in the present experiment had an emission factor (EF_3) of 1.5%, which was similar to the EF_3 value of the urine-only treatment in Chapter Four, which was 1.9%. Applying DCD and urine to the soil in both the present experiment and in the field experiment described in Chapter Four gave an EF_3 of 0.7%. These values are all in line with those published previously (e.g. Dobbie *et al.* 1999, de Klein *et al.* 2003).

Total N_2O emissions were reported to be positively correlated with soil moisture content in Chapter Five of this thesis. Similarly, the trampled soil in this experiment had a higher volumetric water content than the non-trampled soil at all observational points (Figure 6.26). The total N_2O emission was significantly higher from the TU soil (7.20 kg N_20-N ha^{-1}) than it was from the NTU soil (3.48 kg N_2O-N ha^{-1}) (Table 6.3). Many authors have associated high N_2O emission fluxes with high soil moisture (e.g. Luo *et al.* 2007, Beare *et al.* 2009, Monaghan *et al.* 2013).

Simulating animal trampling increased the bulk density of the fine earth fraction of the soil in the present experiment (Table 6.7) ($P<0.05$). Similar findings have been observed by Drewry *et al.* (2000), Di *et al.* (2001), and Ball *et al.* (2012) in finer textured soil, however, at the time of writing, no authors had described physical changes in stony soil in response to animal trampling.

Animal trampling has been shown to reduce the macroporosity of soil, which can impede drainage and water movement (Drewry and Paton 2005). The ability for water to move through a soil is often assessed by measuring the hydraulic conductivity of a soil, and air permeability has been correlated with hydraulic conductivity (Iversen *et al.* 2004, Wells *et al.* 2006, Chief *et al.* 2008b). Simulated animal trampling led to a loss of macropores and subsequent impediment to drainage in the present experiment. Methodological issues did not allow accurate air permeability measurements to be made on the soil. However, when rain fell on the non-trampled soil, it was observed that the water immediately infiltrated the highly permeable, stony soil, while in contrast, the water ponded on the surface of the trampled soil for several hours (Figure 6.28). Ponding of water on the trampled soil after rainfall was observed until the soil was disturbed when an oat crop was sown, 63 days after the soil was trampled.

Figure 6.28: Ponding immediately following rainfall on trampled lysimeters (left), but no ponding on non-tramped lysimeters (right). Photographs taken 54 days after treatments were applied.

In the urine treated lysimeters, simulated animal trampling had a considerable effect on N_2O emissions, because of the decrease in the aeration status of the soil, which led to increased N_2O emissions.

While the surface ponding on the trampled soil after rainfall (Figure 6.) would probably saturate the soil and could favour denitrification (Firestone and Davidson 1989), there was no increase in the abundance of denitrifying *nirS* or *nosZ* gene copies in the trampled soil, when compared with the non-trampled soil (Figure 6.22a, b, Figure 6.23a, b). Where Hamonts *et al.* (2013) found *nirS* and *nosZ* genes to be more abundant in soils with urea than in soils with no urea, in the present study, unexpectedly, both *nirS* and *nosZ* gene copies were, on average, least abundant in the trampled soil

with urine (TU) ($P<0.05$). In Chapter Five of this thesis, denitrifying gene copies were reported to be more abundant in trampled soil at a high soil moisture, than in non-trampled, or drier soil. However, the volumetric soil moisture in the present experiment (Figure 6.26) was still relatively low (25-35%), and more comparable with the low-moisture soil in the laboratory experiment of Chapter Five, where denitrifying gene copies were reportedly least abundant.

Although there was no difference in the abundance of denitrifying gene copies between trampled and non-trampled soils, it was likely that some amount of denitrification was occurring in the trampled soil, and contributing to the N_2O flux. Even after ploughing was simulated, the aggregates in the trampled soil were large and blocky in comparison with those in the non-trampled soil (Figure 6.29), which is supported by Ball *et al.* (2012), who noted an increase in aggregate size and loss of soil structure following simulated trampling. The large size of the trampled aggregates may have prevented oxygen diffusion into the centre of the aggregates, and may have promoted denitrification inside the aggregates of the trampled soil (Hojberg *et al.* 1994). Applying DCD to the trampled soil with urine delayed N_2O emissions by about three months, after which time, the rate of N_2O emission was not significantly different between the TUD and TU treatments (Figure 6.16a, b).

Figure 6.29: Comparison of aggregates in non-trampled soil (left), with aggregates of trampled soil (right) following simulated ploughing.

6.5 Conclusions

Simulated animal trampling substantially decreased the air permeability, and increased the bulk density of the fine earth fraction of the stony Balmoral soil. When urine was applied at a rate of 400 kg N ha^{-1}, the trampled soil emitted 7.20 kg N_2O-N ha^{-1}, which was more than twice the 3.48 kg N_2O-N ha^{-1} emitted by the non-trampled soil with urine. When dairy cow urine was present, applying a nitrification inhibitor, DCD, decreased total N_2O emissions from trampled soil by 44%, but had no significant effect on N_2O emissions when the soil was not trampled. The combination of animal trampling, cow urine, and DCD significantly increased the amount of plant dry matter, and amount of nitrogen removed by an oat crop grown in the months following simulated grazing.

Simulated animal trampling had no statistically significant effect on the abundance of NO_2^- reducing *nirS*, or N_2O reducing *nosZ* gene copies within the soil, as variability between samples was high. The air permeability (AP) of the trampled soil initially exceeded the measurement capacity of the equipment used, which meant it was greater than the AP of the non-trampled soil. However, following soil disturbance from simulated ploughing, there was no statistically significant difference in the AP between trampled and non-trampled soil. Simulated animal trampling had no effect on the matric potential recorded by tensiometers, and peak N_2O emissions were not correlated with the matric potential of the soil, however total N_2O emissions were correlated with soil volumetric water content.

The large, impermeable, soil aggregates that formed when animal trampling was simulated, protected urine-N from leaching, and would have increased the likelihood of aggregate-scale denitrification, which would account for the higher N_2O emission flux from trampled soil.

Chapter Seven – The effect of simulated animal trampling on nitrous oxide emissions, air permeability, and denitrifying gene abundance in soil – a field experiment

7.1 Introduction

Anthropogenic emissions of nitrous oxide (N_2O) have steadily increased since the beginning of the industrial revolution (IPCC 2013). Nitrous oxide is one of the four most important greenhouse gases (GHG) produced by human activity, as it has a global warming potential about 300 times that of carbon dioxide (IPCC 2013). The agricultural sector contributes about half of the total annual GHG emissions in New Zealand (NZ), with more than one third of those emissions being N_2O (Ministry for the Environment 2013). Most of NZ's N_2O emissions occur when grazing animals deposit excreta on soil, and annual N_2O emissions from NZ's agricultural sector have increased by 32% since 1990 (Ministry for the Environment 2014).

To provide animal feed during the winter months when pasture growth is slow, farmers in the Canterbury region of NZ commonly grow high yielding forage crops, which are grazed in-situ. When dairy cows are taken to graze the high yielding crops, soil trampling damage and concentrated urine deposition can occur in the cool, wet, environment (Drewry and Paton 2005) (Figure 7.1). Both urine deposition and animal trampling produce soil conditions conducive to denitrification and elevated N_2O emissions (Menneer *et al.* 2005, van Groenigen *et al.* 2005b, Ball *et al.* 2012).

Most N_2O from an agricultural setting is produced during the microbial processes of nitrification and denitrification in soil (Firestone and Davidson 1989, Groffman *et al.* 2006). Although N_2O emissions from forage crops have been reported from slow-draining fine textured soil (e.g. Smith *et al.* 2008b, Thomas *et al.* 2008, Monaghan *et al.* 2013), no authors have described N_2O emissions from forage crops grown in stony soil. Furthermore, no authors have described N_2O emissions from forage crops in relation to the denitrifying microbial population. Consequently, there is currently insufficient information to determine the contribution of N_2O from winter forage grazing by dairy cows when preparing national GHG inventories (Ministry for the Environment 2011).

The results reported in Chapter Six of this thesis, showed that when dairy cow urine was present, emissions of N_2O from trampled soil were more than twice that from non-trampled soil. Ponding of surface water and a decrease in the air permeability (AP) of the trampled soil suggested that the interior of the large soil aggregates were anaerobic, and denitrification might have occurred

in the trampled soil, leading to the high N_2O flux. However, the low permeability of the trampled soil was beyond the range of measurement of the available equipment, preventing accurate measurements to be made. Also, it was not clear if native soil N was being released as N_2O, or if the urine-N was the dominant N_2O source. Additionally, measurements of the abundance of denitrifying gene copies in Chapter Six were highly variable, and no statistically significant differences in the denitrifying microbial population between trampled and non-trampled soil were found.

The aims of the present experiment, therefore, were to (i) overcome the methodological issues encountered when measuring the AP of the soil, and (ii) to reduce the variability in denitrifying gene abundance measurements by using an improved DNA extraction technique and increasing the number of treatment replicates. Nitrous oxide emissions were quantified, and, using stable isotopes of N, the proportion of urine-derived N_2O was assessed. The bulk density of the soil, and the mineral N concentration in the soil were also quantified.

Figure 7.1: **Heavy trampling damage to soil following winter grazing of forage kale by dairy cows at Lincoln University's Ashley Dene Research farm. Note spade for scale.**

7.2 Methods

7.2.1 Soil and climate

Soil

A Balmoral stony silt loam, classified as Pallic Orthic Brown Soil (Hewitt 2010); Udic Haplustept loamy skeletal (Soil Survey Staff 2010), typical of soils used for dairy forage grazing during winter in the Canterbury region, South Island of New Zealand, was used in this study. Developed from gravelly glacial outwash alluvium and loess, the Balmoral soil consists of a thin, sandy silt loam top soil containing about 30% stones. Below the top soil, the stone and sand content increases substantially, making this soil very free draining (Figure 7.2).

Figure 7.2: A Balmoral soil at Lincoln University's Ashley Dene Farm. Note high stone content throughout the soil profile.

Forty soil blocks were established in a field facility at Lincoln University (43°39'04"S, 172°28'03"E) (refer to Chapter Six for a detailed description). The soil from a previous experiment (Chapter Six) was discarded, and fresh soil collected during May 2013 from Lincoln University's Ashley Dene farm (43°38'55"S, 172°20'39"E), and placed in the rings (Figure 7.3). Twenty soil blocks were randomly set aside for destructive soil sampling and bulk density analysis, while the remaining twenty soil blocks were used to measure N_2O emissions, and the air permeability of the undisturbed soil.

Climate

The average annual rainfall in the Canterbury region is about 650 mm, and the average annual temperature about 12.1 °C. Water input was maintained at the 75th percentile of the regional average using hand held sprinklers when required. Rainfall at the experimental site was measured by a tipping bucket rain gauge (model OSK 15180-T, Ogawa Seiki, Japan), while soil and air temperature at the site were measured by Campbell Scientific CS107 sensors.

Figure 7.3: Experimental site used during 2013, showing soil rings filled with soil.

7.2.2 Treatments

Four treatments, each with five replicates, were randomly allocated to the lysimeters on 22nd July 2013 (table 7.1). The soil in the trampled soil blocks was trampled using cow hoof simulation equipment. The hoof simulation equipment comprised a commercially available post hole compactor, with a groove cut in the lower surface to mimic the shape of a cow hoof. A foot peg was welded to the shaft of the trampling device to allow the user to apply force to the shaft. A combined mass of 70 kg for the user and device would apply about 230 kPa, similar to the pressure exerted by an adult cow hoof (Di *et al.* 2001).

The surface of the soil was trampled for a period of 5 minutes per 0.2 m^2 soil block, until the soil visually represented field observations of soil under forage crops grazed by dairy cows during winter (Figure 7.4). Fresh urine was collected from non-lactating dairy cows that were feeding on kale, and analysed for total N concentration using an Elemental Analyser (Elementar Vario MAX CN). The urine was enriched with ^{15}N labelled urea at a rate of 10 atom%, and then standardised to 4.0 g N L^{-1} by adding a small amount of de-ionised water, and applied to the trampled + urine (TU) and non-trampled + urine (NTU) soil blocks at a rate equivalent to 400 kg N ha^{-1}.

Figure 7.4: A soil block following simulated trampling and urine application.

Table 7.1: Details of the treatments applied to the soil.

Treatment name[#]	Trampling (yes/no)	Urine-N (kg N ha^{-1})
T	Y	0
TU	Y	400
NT	N	0
NTU	N	400

[#] 'T' indicates simulated trampled treatments, 'NT' indicates no trampling, 'U' indicates urine was applied.

7.2.3 Nitrous oxide emissions

A closed chamber method, similar to that of Hutchison & Mosier (1981), was used to determine N_2O emissions. A water filled trough was sealed to the top of each soil ring to provide a gas tight seal for the metal and polystyrene chamber used during sampling. At each sampling time, the chambers were placed on top of the lysimeters for a total of 40 minutes, with three samples taken 20 minutes apart, i.e. t=0, t=20, and t=40 minutes. Samples were taken through a rubber septum located in the top of each chamber using a 60 ml syringe and hypodermic needle, and placed in over pressurized 6 ml glass vials. Samples for N_2O analysis were taken between 12:00 and 14:00 hours at each sampling event. Nitrous oxide concentration was analysed using gas chromatograph (SRI 8610GC, SRI Instrument, C.A., USA). A separate sample of headspace gas was taken on nine occasions, and placed in over pressurized 12 ml glass vials for ^{15}N analysis using a Continuous Flow Isotope Ratio Mass Spectrometer (20-20 Secron™ Ltd., PDZ Europa Ltd, Crewe, CWI6ZA, UK).

7.2.4 Soil mineral N

A sample of soil consisting of three cores, 7.5 cm deep, was taken from each of the 20 soil rings set aside for destructive sampling on six occasions during the course of the experiment. Each of the 20 samples were thoroughly mixed, and five grams of soil was placed in a centrifuge tube before adding 25 ml of 2 M KCl. The tubes were then placed in a rack and shaken for 1 hour. After shaking, the solution was centrifuged for 10 minutes, before being filtered through Whatman #42 (or equivalent) filter paper. The filtrate was frozen until analysis for NH_4^+ and NO_3^- concentration using a flow injection analyser (Tecator Inc. Sweden). The soil moisture content was calculated gravimetrically by drying approximately 10 g of soil at 105°C for a minimum of 24 hours.

7.2.5 Denitrifying microbial gene abundance

Additional samples of soil for assessing denitrifying microbial gene abundance were taken at the same times as the samples were taken for mineral N and DCD assays. The abundance of the NO_2^- reductase genes *nirS* and *nirK*, and the N_2O reductase gene *nosZ*, were analysed from soil samples taken from all four treatments. Preliminary work identified a new DNA extraction kit that had demonstrated greater DNA extraction efficiency compared with the kit used in Chapters Five and Six (Macherey-Nagel, Düren, Germany). Approximately five grams of soil was packed into 6 ml plastic vials and stored at -80°C until analysis, using the methods described by Di *et al.* (2014) and Robinson *et al.* (2014) (Table 7.2). Briefly, DNA was extracted from 0.25 g frozen soil using NucleoSpin® Soil Kits (Macherey-Nagel, Düren, Germany) in accordance with the manufacturer's instructions. Extracted DNA was eluted with 100 µL Buffer SE, and stored at -20°C until analysis (Macherey-Nagel, Düren, Germany). Quant-iT™ dsDNA BR assay kits, a Qubit fluorometer (Life Technologies, Auckland, New Zealand), and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Montchanin, USA), were used to determine the concentration and quality of the extracted DNA. For real-time qPCR analysis, samples were prepared using a CAS-1200 Robotic liquid handling system (Corbett Life Science, BioStrategy, Auckland, New Zealand), and analysed using a Rotor-Gene™ 6000 real-time rotary analyser (Corbett Life Science, BioStrategy, Auckland, New Zealand).

7.2.6 Physical measurements

Bulk Density

On 13th November 2013, samples were taken to determine the bulk density of the soil from un-disturbed portions of each soil sampling ring, using a sand replacement method (Equation 7.1):

$$\mathrm{P_B} = \frac{M_{\mathrm{soil}}}{\mathrm{V_{soil}}} \qquad (7.1)$$

where P_B is the soil bulk density (Mg/m^3), M_{soil} is the mass of soil, and V_{soil} is the volume of soil, where $V_{soil} = M_{sand}/P_{sand}$, where M_{sand} is the mass of sand, and P_{sand} is the density of the sand.

Samples were taken from ten trampled, and ten non-trampled soil rings. First, the surface of the soil was made flat, and then approximately 500 ml soil was extracted and placed in a paper bag. A plastic bag was then placed inside the cavity created by the excavated soil, into which dry sand of a known bulk density was carefully poured until the sand filled the void in the soil. The soil was dried in an oven at 105°C for a minimum of 24 hours, and weighed.

Table 7.2: Primer pairs and PCR conditions used in real-time qPCR analysis (Di *et al.* 2014).

Target group	Primer name	Sequence (5'-3')	Length of amplicon (bp)	Primer final concentration (nM)	Thermal profile	Amplification efficiency (R^2>0.99) (%)	References
nirS	Cd3aF R3cd	5'-GTSAACGTSAAGGARACSGG-3' 5'-GASTTCGGRTGSGTCTTGA-3'	410	750	95 °C for 2 min - x 1 cycle; 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, 85 °C for 20 s - x 40 cycles;	93-95	(Michotey *et al.* 2000, Throback *et al.* 2004)
nirK	FlaCu R3Cu	5'-ATCATGGTSCTGCCGCG-3' 5'-GCCTCGATCAGRTTGTGGTT-3'	474	780	95 °C for 2 min - x 1 cycle; 95 °C for 20 s, 63 °C for 30 s, 72 °C for 30 s, 85 °C for 15 s - x 40 cycles;	98-100	(Hallin and Lindgren 1999)
nosZ (I)	NosZ-F NosZ1622R	5'-CGYTGTTCMTCGACAGCCAG-3' 5'-CGSACCTTSTTGCCSTYGCG-3'	424	750	95 °C for 2 min – x 1 cycle; 95 °C for 20 s, 58°C for 30 s, 72 °C for 30 s, 85 °C for 15 s - x 40 cycles;	94-99	(Kloos *et al.* 2001, Throback *et al.* 2004)
nosZ (II)	nosZ-II-F nosZ-II-R	5'-CTIGGICCIYTKCAYAC-3' 5'-GCIGARCARAAITCBGTRC-3'	698	1000	95 °C for 2 min – x 1 cycle; 95 °C for 30 s, 54°C for 30 s, 72 °C for 40 s, 85 °C for 15 s - x 40 cycles;	76-81	(Jones *et al.* 2013)

The fine earth fraction of the soil was separated from the stones by sieving the dried samples using a 2 mm mesh sieve. The stones were washed, dried, and weighed to determine their relative contribution to total soil mass. The volume of stones was calculated by placing stones in a known volume of water and observing the mass of water displaced by the stones (density of water assumed to be 1 g cm^{-3}).

Air permeability

The air permeability (AP) of the soil was measured on each of the twenty un-disturbed soil rings used for N_2O emission measurements, giving ten trampled, and ten non-trampled replicates. A rubber channel was sealed to the top of each soil ring, to which a steel lid was clamped to provide an air tight seal. To create a flow of air through the soil, a cylinder of dry compressed air was connected via a regulator to a variable flow meter (0-60 L min^{-1} capacity), which was connected to the steel lid (see Chapter Six for a detailed explanation). A tube from the metal lid connected to a universal pressure module (GE Druck, Leicester, UK), which communicated to a pressure indicator (DPI 150, GE Druck, Leicester, UK) with a range of 1 bar (Figure 7.5). To calculate the AP, the regulator on the gas cylinder was manipulated until steady flows of 5, 10, 20, 30, and 50 L min^{-1} through the soil ring were reached. The pressure module was zero-calibrated to the ambient air pressure, and pressure readings taken at each of the four flow rates.

The AP of the soil was calculated using Equation 7.2 and 7.3:

$$k_a = \frac{\eta Q}{\Delta P A} \tag{7.2}$$

where k_a is the air permeability (m^2), η is the gas viscosity (assumed to be 1.85 x 10^{-5} P), Q is the volumetric flow rate ($m^3\ s^{-1}$), ΔP is the change in pressure between both sides of the soil (Pa), and A is a shape factor (m) (Equation 7.3).

$$A = 0.4862\left(\frac{D}{H}\right) - 0.0287\left(\frac{D}{H}\right)^2 + 0.1106 \tag{7.3}$$

where D is the diameter of the soil ring, and H is the height of the soil within the soil ring.

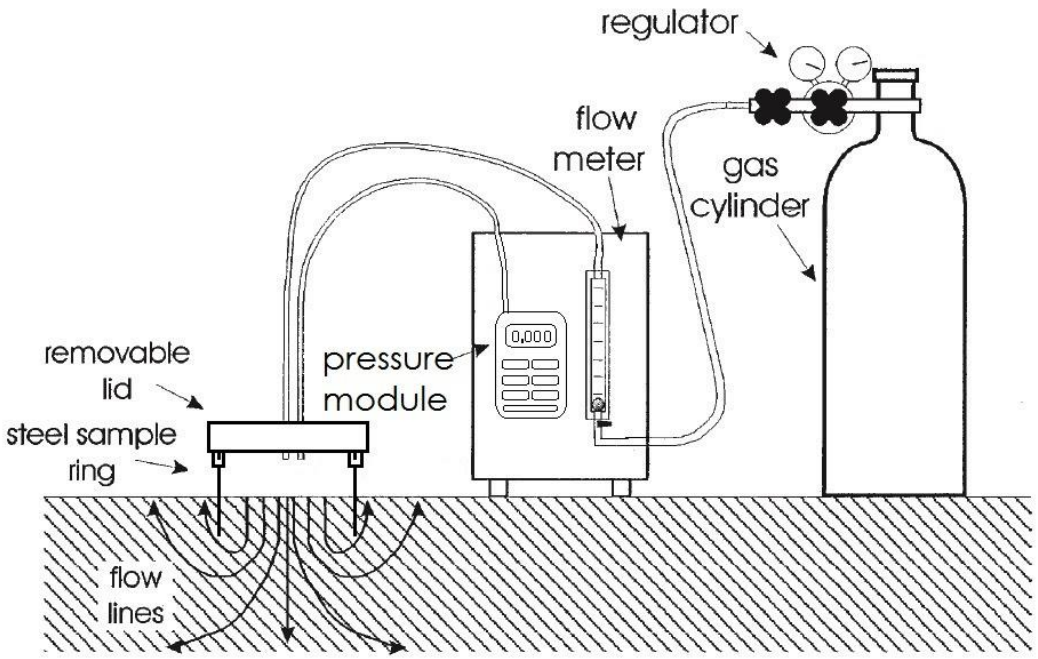

Figure 7.5: Apparatus for measuring the air permeability of soil (modified from Iversen *et al.* (2001)).

7.2.7 Data analysis

Nitrous oxide emission rates were calculated linearly from the increase in concentration of N_2O between the first and second gas samples taken at each sampling event. Daily N_2O fluxes were then calculated on the assumption that the hourly flux represented the average hourly flux for that day. Total N_2O emissions were calculated by integrating the daily emission fluxes.

The emission factor (EF_3), or proportion of applied N that was emitted as N_2O was calculated using Equation 7.4 (de Klein *et al.* 2003):

$$EF(\%) = \frac{N_2O\text{-}N\ total\ (treatment) - N_2O\text{-}N\ total\ (control)}{UrineN\ (applied)}\ x\ 100 \quad (7.4)$$

where *EF(%)* is the emission factor, *N_2O-N total (treatment)* is the cumulative total N_2O emitted from a urine treatment, *N_2O-N total (control)* is the cumulative total N_2O emitted from the comparative no-urine treatment, and *UrineN (applied)* is the amount of N added as urine.

The proportion of ^{15}N in the N_2O derived from the labelled urine-N was calculated by subtracting the background concentration of ^{15}N in the atmosphere, and multiplying the resultant concentration by ten to account for the 10 atom% enrichment.

The average concentration of NH_4^+, NO_3^-, and abundance of gene copies throughout the duration of the experiment were determined by taking the average of the daily concentration between sampling events.

In air permeability calculations, a linear correlation (R^2=0.99) was observed between air flow rates and chamber pressures, and so mean values of pressure and flow from each sample were used.

Least significant differences and P-values were calculated following analysis of variance using the GenStat software package (version 16, VSN International Ltd, UK). Total N_2O, soil mineral N concentration, and AP required a log transformation to determine differences between treatments.

7.3 Results

7.3.1 Climate

During the monitoring period, 156 mm of rain fell, which was supplemented with 203 mm of simulated rainfall (Figure 7.6). The mean daily air temperature ranged from 4.8°C to 18.9°C (Figure 7.7).

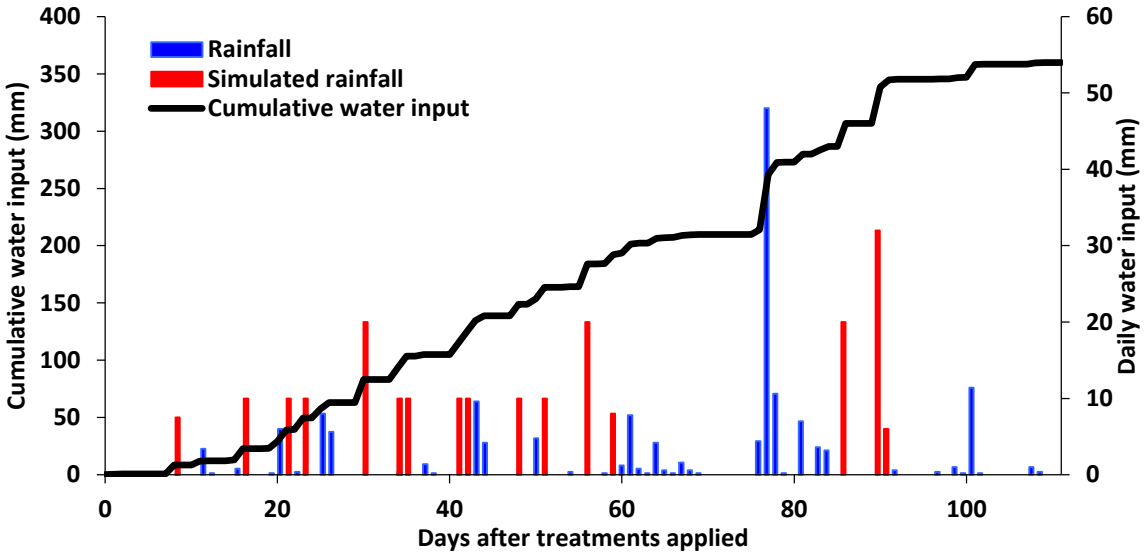

Figure 7.6: **Cumulative and daily water input.**

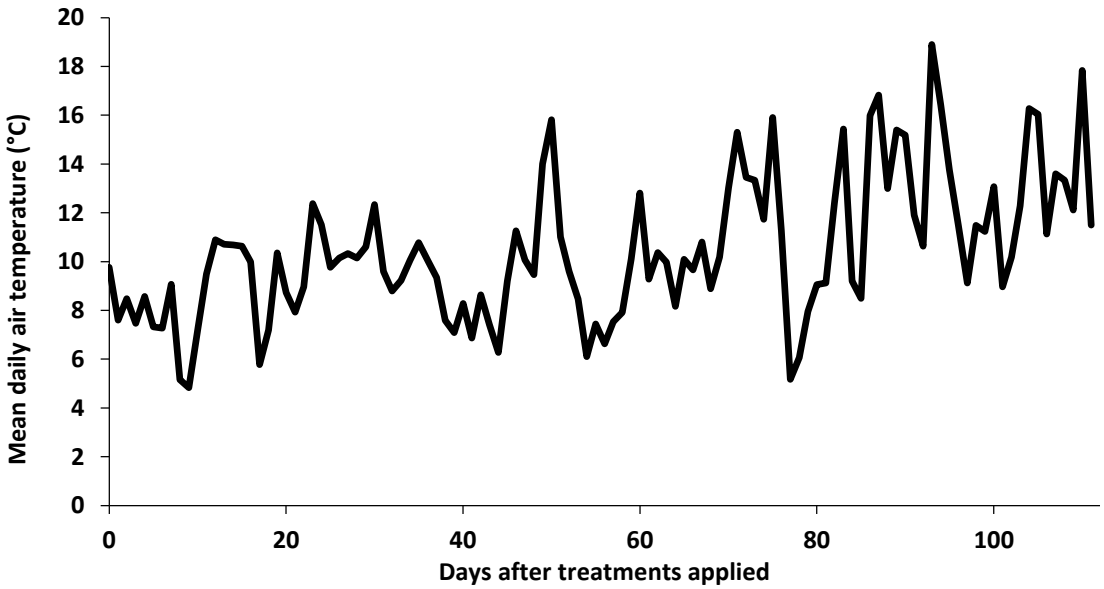

Figure 7.7: **Mean daily air temperature at the experimental site during the monitoring period.**

7.3.2 Nitrous oxide emissions

Peak N_2O emission flux

The highest N_2O emission peaks were produced by the trampled soil with urine (TU), 42, 52, and 63 days after treatments were applied (Figure 7.8). The three peaks ranged from 135 to 151 g N_2O-N ha^{-1} day^{-1}, and were not significantly different. Of the remaining treatments, the trampled soil with no urine (T) produced the next highest N_2O emission peak, seven days after treatments were applied, when a flux of 44 g N_2O-N ha^{-1} day^{-1} was observed. The highest peak flux from the non-trampled soil with urine (NTU) was 21 g N_2O-N ha^{-1} day^{-1}, which occurred 27 days after treatments were applied. The peak flux from the non-trampled soil without urine (NT) was 25 g N_2O-N ha^{-1} day^{-1} which occurred 86 days after treatments were applied. The peak N_2O fluxes from the NT and NTU treatments were not statistically different.

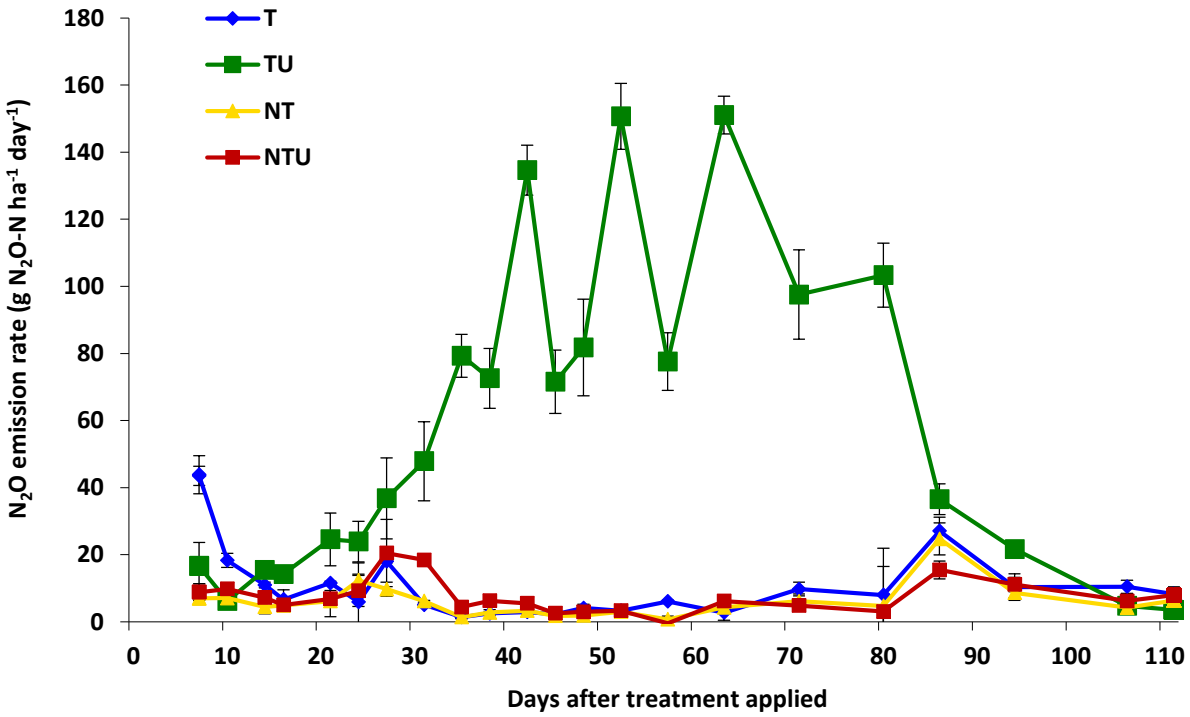

Figure 7.8: **Daily N_2O emission flux from all treatments. Error bars represent one standard error of the mean.**

Total N_2O emissions

Over the 113 day measurement period, the trampled soil with urine (TU) emitted 6.33 kg N_2O-N ha^{-1}, which was significantly more than all other treatments ($P<0.01$) (Figure 7.9,Table 7.3). The non-trampled soil with urine (NTU) emitted 0.82 kg N_2O-N ha^{-1}, which was not significantly

different from the non-trampled soil without urine (NT), which emitted 0.69 kg N_2O-N ha^{-1}, or the trampled soil without urine (T), which emitted 1.24 kg N_2O-N ha^{-1} (Figure 7.9,Table 7.3).

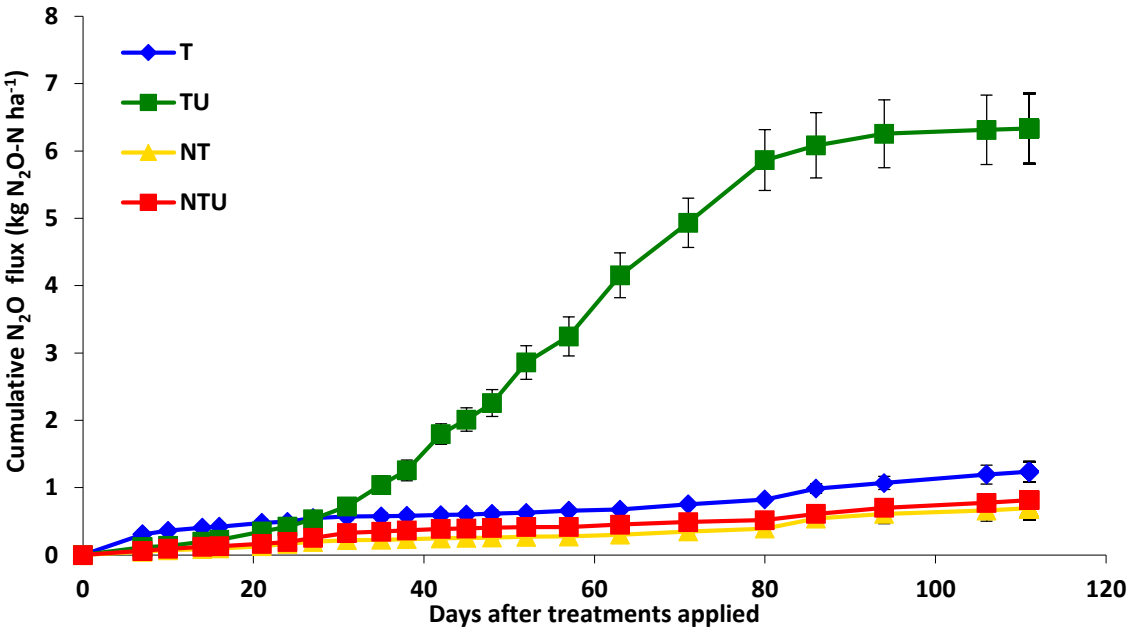

Figure 7.9: **Cumulative N_2O emissions from all treatments. Error bars represent one standard error of the mean.**

Table 7.3: **Log transformed total N_2O emissions with back transformed (BT) means, and proportion of applied N emitted as N_2O (EF_3) from all treatments during the period 23rd June to 11th November 2013.**

Treatment	Total emissions (kg N_2O-N ha^{-1})		EF_3 (%)
	Log_{10} N_2O	BT mean	
T	3.08	1.24	-
TU	3.80	6.33	1.3
NT	2.79	0.69	-
NTU	2.90	0.82	0.03
LSD# (0.05)	0.19	-	-

#Least significant difference ($P < 0.05$).

^{15}N enrichment

The N_2O emitted by the trampled (TU) soil had a higher proportion of ^{15}N than the non-trampled (NTU) soil, which signified a larger proportion of the N_2O emitted by the TU soil was derived from the added urine than the NTU soil (Figure 7.10). On the 48th day after treatments were applied, urine-N represented 56% of the N_2O emitted by the trampled soil, which was the maximum ^{15}N concentration observed during the times ^{15}N samples were analysed. In comparison, a maximum of 5.6% of the N_2O emitted by the non-trampled soil was derived from urine (Figure 7.10). Consequently, almost none of the urine-N was emitted as N_2O from the NTU soil, whereas the proportion of urine-N emitted as N_2O from the trampled soil increased at a linear rate (R^2=0.91) during the first 48 days after urine was applied to the soil (Figure 7.10).

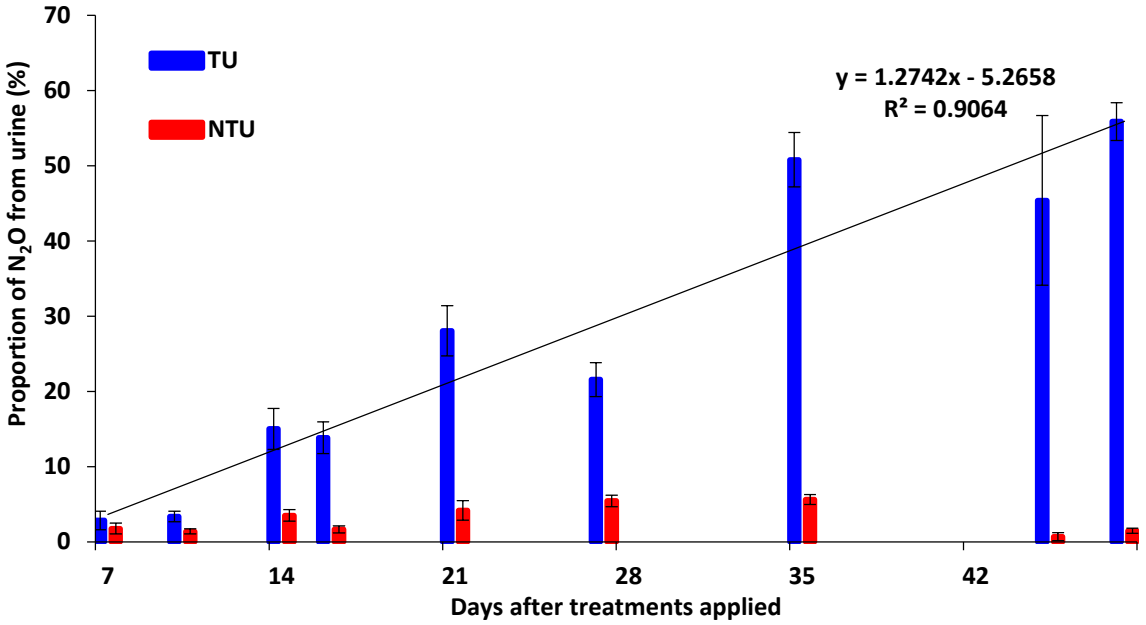

Figure 7.10: **Proportion of urine derived N_2O emitted by the trampled and non-trampled soil, as determined by enrichment of cow urine with ^{15}N labelled urea. Error bars represent one standard error of the mean.**

The rate of ^{15}N enrichment of the urine (10 atom%) was not high enough to quantify N_2 emissions. However, the concentration of ^{15}N in the N_2 collected from the headspace of the chambers was higher from the trampled soil with urine (TU) than the trampled soil without urine (T) on days 21, 27, 35, 45, and 48, and higher than the non-trampled soil with urine (NTU) on days 21, 27, 45, and 48 ($P<0.05$) (Figure 7.11). The concentration of ^{15}N in the N_2 from the NTU soil was not significantly different from the NT soil at any time it was measured (Figure 7.11).

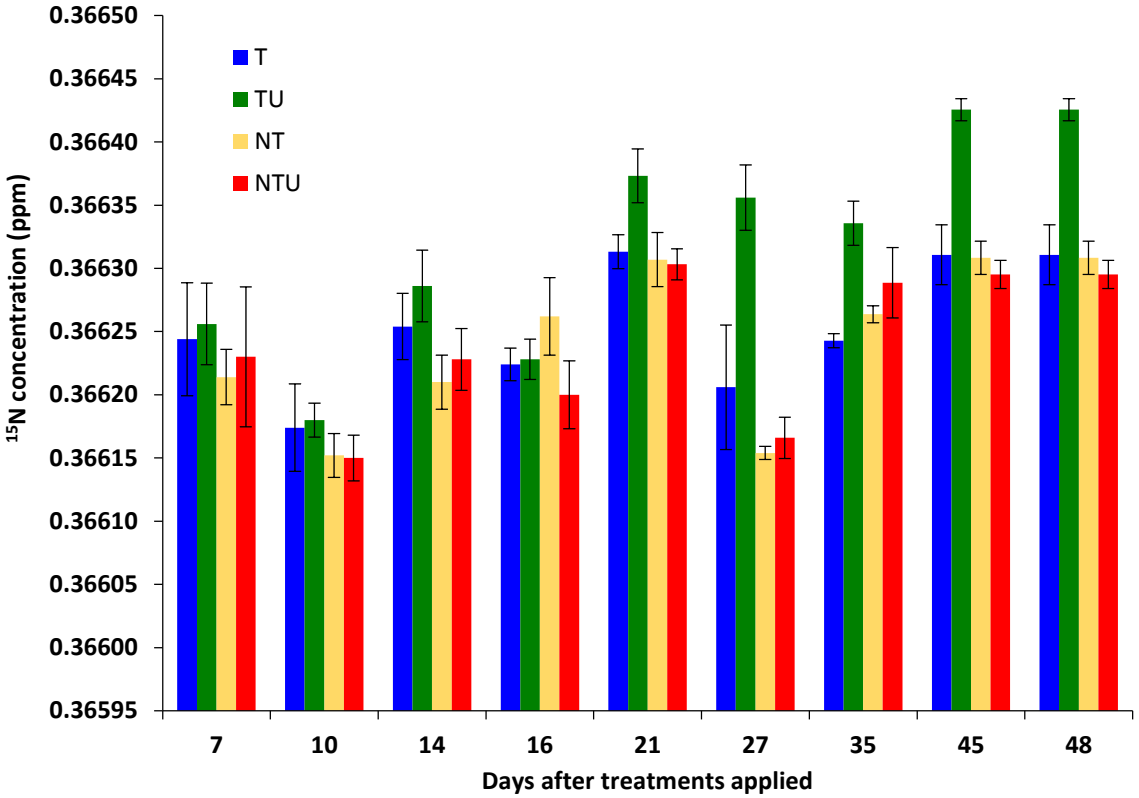

Figure 7.11: **Concentration of ^{15}N emitted as N_2 from the soil during the first 48 days of the experiment. Error bars represent one standard error of the mean.**

7.3.3 Soil mineral N

Ammonium

The peak NH_4^+ concentration of 677 mg NH_4-N kg $soil^{-1}$ was found in the trampled soil with urine (TU), which occurred ten days after treatments were applied ($P<0.01$) (Figure 7.12a). At the same time, the peak NH_4^+ concentration in the non-trampled soil with urine (NTU) was 155 mg NH_4-N kg $soil^{-1}$, which was higher than the treatments where no urine was applied, but significantly less than the TU soil ($P<0.05$). The peak concentration of NH_4^+ in the non-trampled soil without urine (NT) was 7 mg NH_4-N kg $soil^{-1}$, which was not significantly different to that of the trampled soil without urine (T), where the peak NH_4^+ concentration was 12 mg NH_4-N kg $soil^{-1}$.

The average concentration of NH_4^+ throughout the duration of the experiment was highest in the trampled soil with urine (TU), followed by the non-trampled soil with urine (NTU). The average concentration of NH_4^+ in the treatments that did not receive urine were not significantly different (Table 7.4).

Nitrate

The peak NO_3^- concentration in the trampled soil with urine (TU) was 103 mg NO_3-N kg $soil^{-1}$ (Figure 7.12b), which occurred 111 days after treatments were applied. The peak NO_3^- concentration in the non-trampled soil with urine (NTU) of 71 mg NO_3-N kg $soil^{-1}$ was not significantly different to the TU, however it occurred earlier, 41 days after treatments were applied (Figure 7.12b). The peak soil NO_3^- concentration in the treatments where no urine was applied occurred at the end of the experiment, 111 days after treatments were applied (Figure 7.12b), and were significantly lower than the urine treatments.

The average concentration of NO_3^- throughout the duration of the experiment was highest in the trampled soil with urine (TU), followed by the non-trampled soil with urine (NTU) ($P<0.05$) (Table 7.4). The average soil NO_3^- concentration was not significantly different between the treatments that did not receive urine (Table 7.4).

Table 7.4: Log transformed average soil mineral nitrogen concentration with back transformed means over the duration of the experiment, from 21st June to 25th October 2012.

Treatment	Average NH_4^+ (mg NH_4-N kg $soil^{-1}$)		Average NO_3^- (mg NO_3-N kg $soil^{-1}$)	
	Log_{10} NH_4-N	BT mean	Log_{10} NO_3-N	BT mean
T	0.87	7.4	1.02	10.8
TU	2.19	155.8	1.76	58.1
NT	0.78	6.0	1.13	14.2
NTU	1.45	28.7	1.55	37.5
LSD# (0.05)	0.10	-	0.19	-

#Least significant difference ($P < 0.05$).

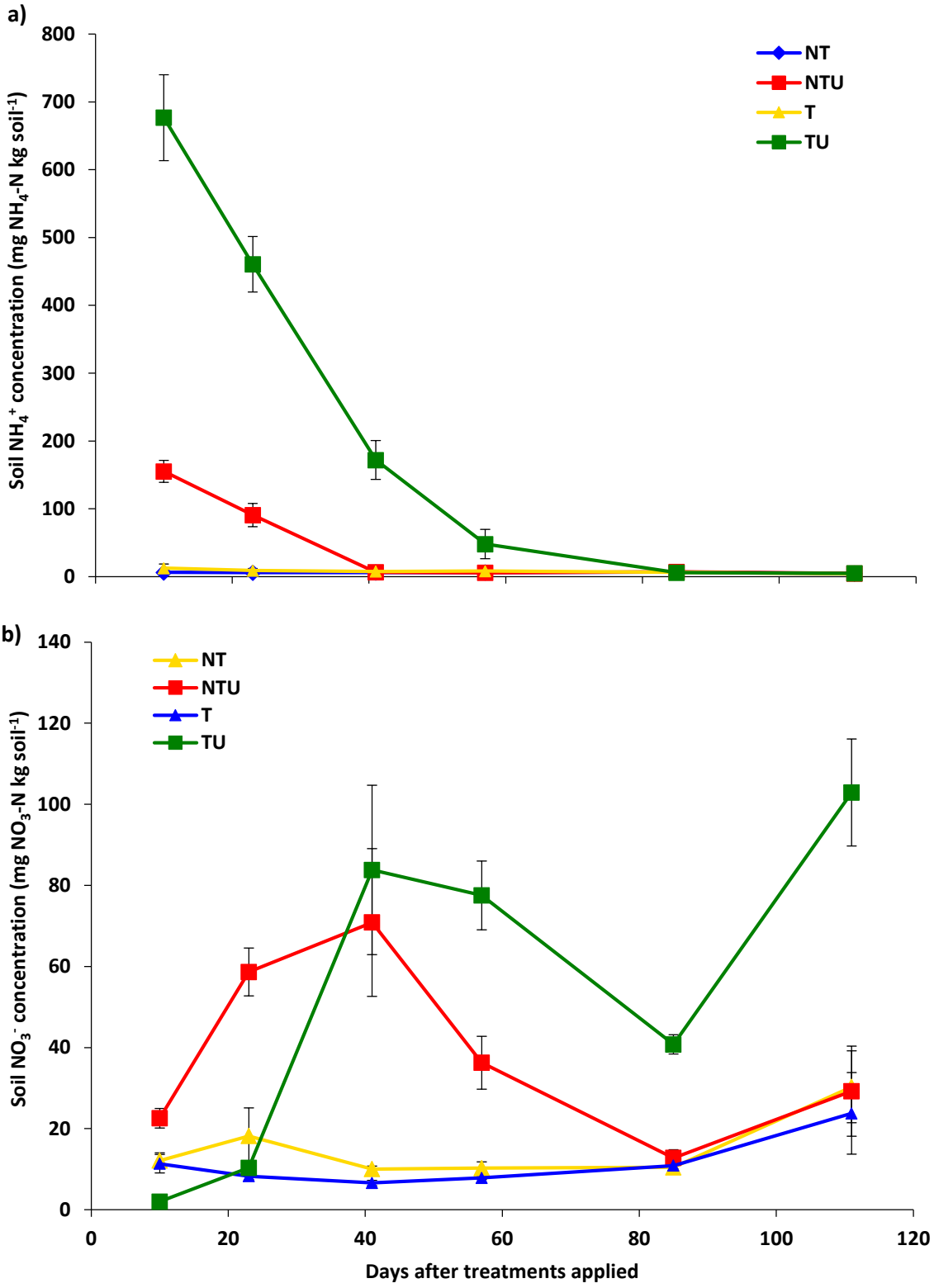

Figure 7.12: **(a) Ammonium, and (b) nitrate concentration in the soil during the experimental period. Error bars represent one standard error of the mean.**

7.3.4 Denitrifying microbial gene abundance

Throughout the duration of the experiment, the abundance of NO_2^- reducing *nirS* gene copies were, on average, higher in the non-trampled soil with urine (NTU) than in all other treatments ($P<0.05$) (Table 7.5). The average abundance of *nirS* gene copies were not significantly different between the other treatments. The peak *nirS* gene copy numbers for all treatments were observed at the end of the experiment, 111 days after treatments were applied (Figure 7.15a). The abundance of *nirS* gene copies was not statistically different at the start of the experiment compared with the end of the experiment in the non-trampled soil with (NTU), or without dairy cow urine (NT) (Figure 7.13a). However, the abundance of *nirS* gene copies in the TU soil increased from 1.67×10^5 to 3.12×10^5 copies g soil^{-1} over the duration of the experiment ($P<0.05$), similarly the abundance of *nirS* gene copies was higher in the trampled soil without urine at the end of the experiment compared with the start (Figure 7.13a).

Overall, dairy cow urine slightly increased the average abundance of NO_2^- reducing *nirK* gene copies in both trampled, and non-trampled soil ($P<0.05$) (Table 7.5). However, simulated animal trampling had no significant effect on the average abundance of *nirK* gene copies ($P=0.72$) (Figure 7.15b). When urine was present (TU and NTU), the abundance of *nirK* gene copies were not significantly different between the start, and the end of the experiment. However, in the non-trampled soil where no urine was applied (NT), the abundance of *nirK* gene copies decreased during the experimental period ($P<0.05$) (Figure 7.13b).

Dairy cow urine increased the average abundance of N_2O reducing *nosZ* clade I (*nosZ-I*) gene copies in trampled soil ($P<0.01$), but not in non-trampled soil ($P>0.05$) (Table 7.5). Simulated animal trampling increased the average *nosZ-I* gene copy abundance with, and without, dairy cow urine (Table 7.5). Consequently, the average abundance of *nosZ-I* gene copies were highest in the TU soil, and lowest in the NT soil (Table 7.5). There was no significant change in *nosZ-I* abundance in non-trampled soil during the experiment (Figure 7.16a). In contrast, the abundance of *nosZ-I* gene copies was considerably higher at the end of the experiment than at the start in both the T ($P<0.05$) and TU soil ($P<0.01$) (Figure 7.14a).

Simulated animal trampling increased the average abundance of *nosZ* clade II (*nosZ-II*) gene copies in soil with, and without dairy cow urine (P<0.01) (Table 7.5, Figure 7.16b). Dairy cow urine had no significant effect on the average abundance of *nosZ-II* gene copies in trampled or non-trampled soil (Table 7.5). The abundance of *nosZ-II* gene copies was higher in the T and TU soil at the end of the experiment than it was at the start of the experiment (P<0.01) (Figure 7.14b). Marginally more *nosZ-II* gene copies were found in the NT soil at the end of the experiment than at the start (P<0.05), but no change in *nosZ-II* gene copies was observed between the start and the end of the experiment in the NTU soil (Figure 7.14b).

Table 7.5: Average abundance of microbial denitrifying NO_2^- reductase *nirS* and *nirK*, and N_2O reductase *nosZ* clade I and clade II gene copies in the soil over the duration of the experiment.

Treatment	Average *nirS* abundance (copies g soil^{-1})	Average *nirK* abundance (copies g soil^{-1})	Average *nosZ-I* abundance (copies g soil^{-1})	Average *nosZ-II* abundance (copies g soil^{-1})
T	2.21×10^5	3.11×10^4	5.61×10^3	1.54×10^4
TU	2.34×10^5	3.51×10^4	8.93×10^3	1.55×10^4
NT	2.39×10^5	3.04×10^4	4.62×10^3	1.22×10^4
NTU	2.59×10^5	3.45×10^4	5.14×10^3	1.29×10^4
LSD# (0.05)	2.0×10^4	3.3×10^3	9.2×10^2	1.9×10^3

#Least significant difference (P < 0.05).

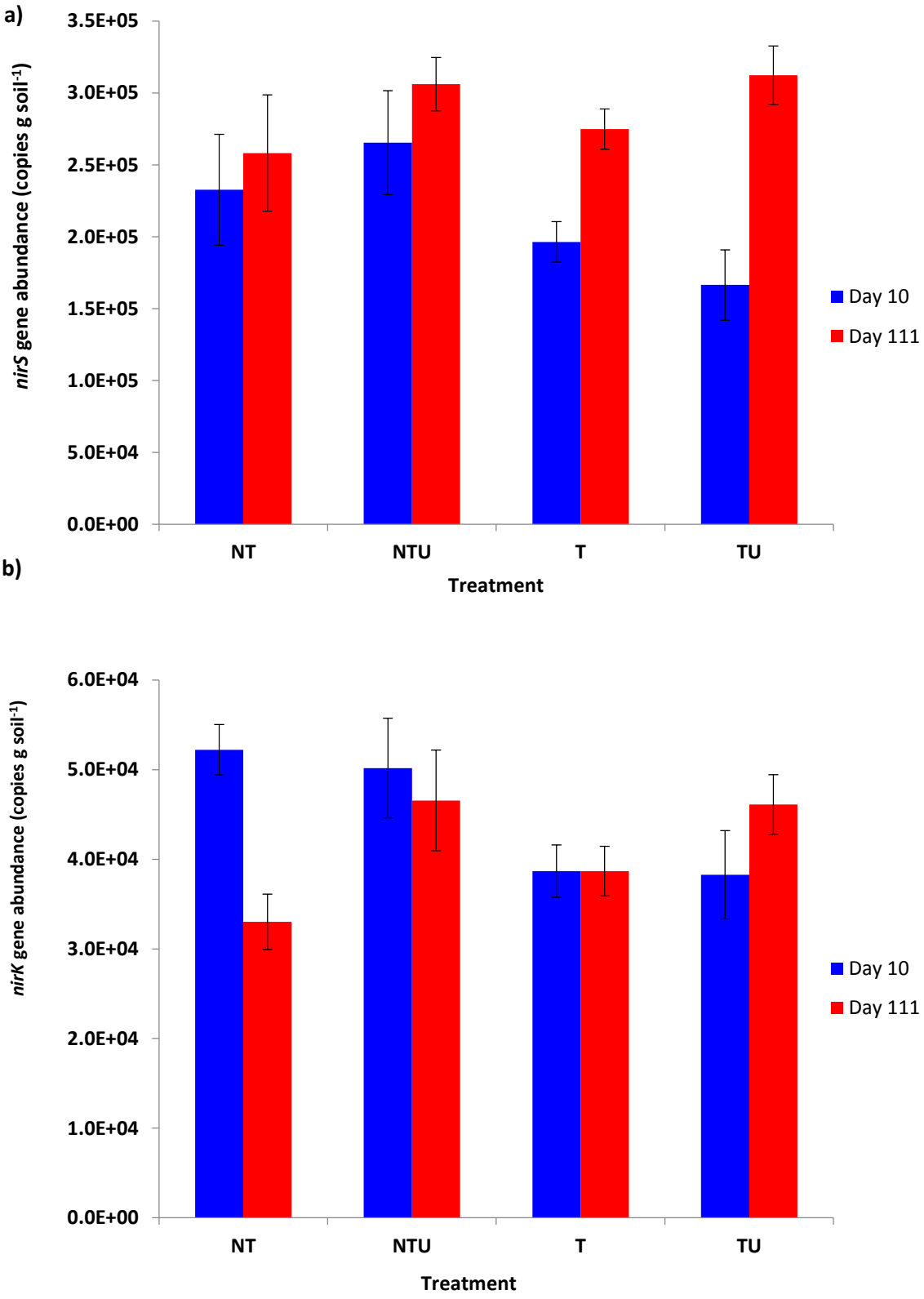

Figure 7.13: **Abundance of (a) *nirS*, and (b) *nirK* gene copies 10, and 111 days after treatments were applied.**

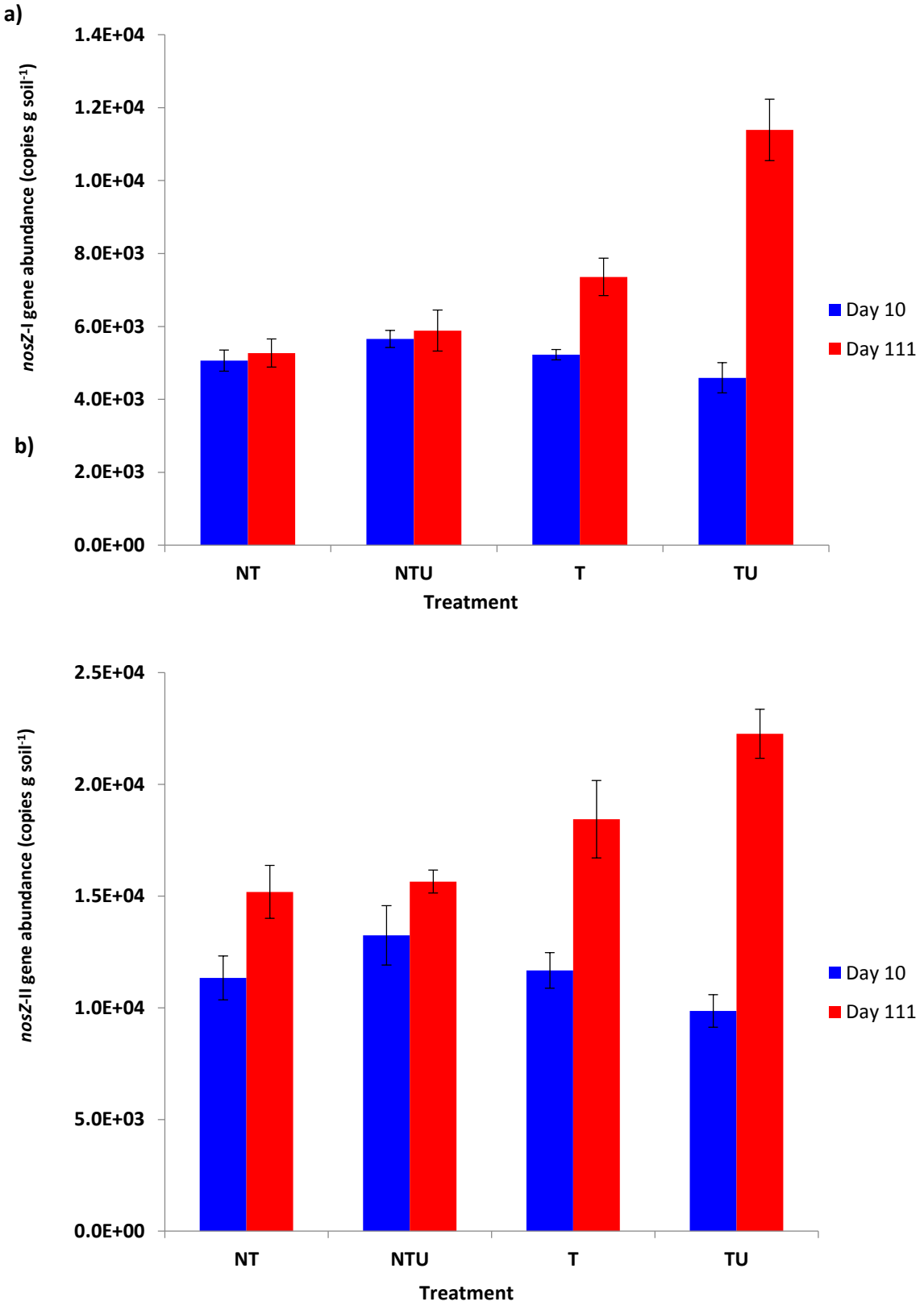

Figure 7.14: **Abundance of (a) *nosZ*-I, and (b) *nosZ*-II gene copies 10, and 111 days after treatments were applied.**

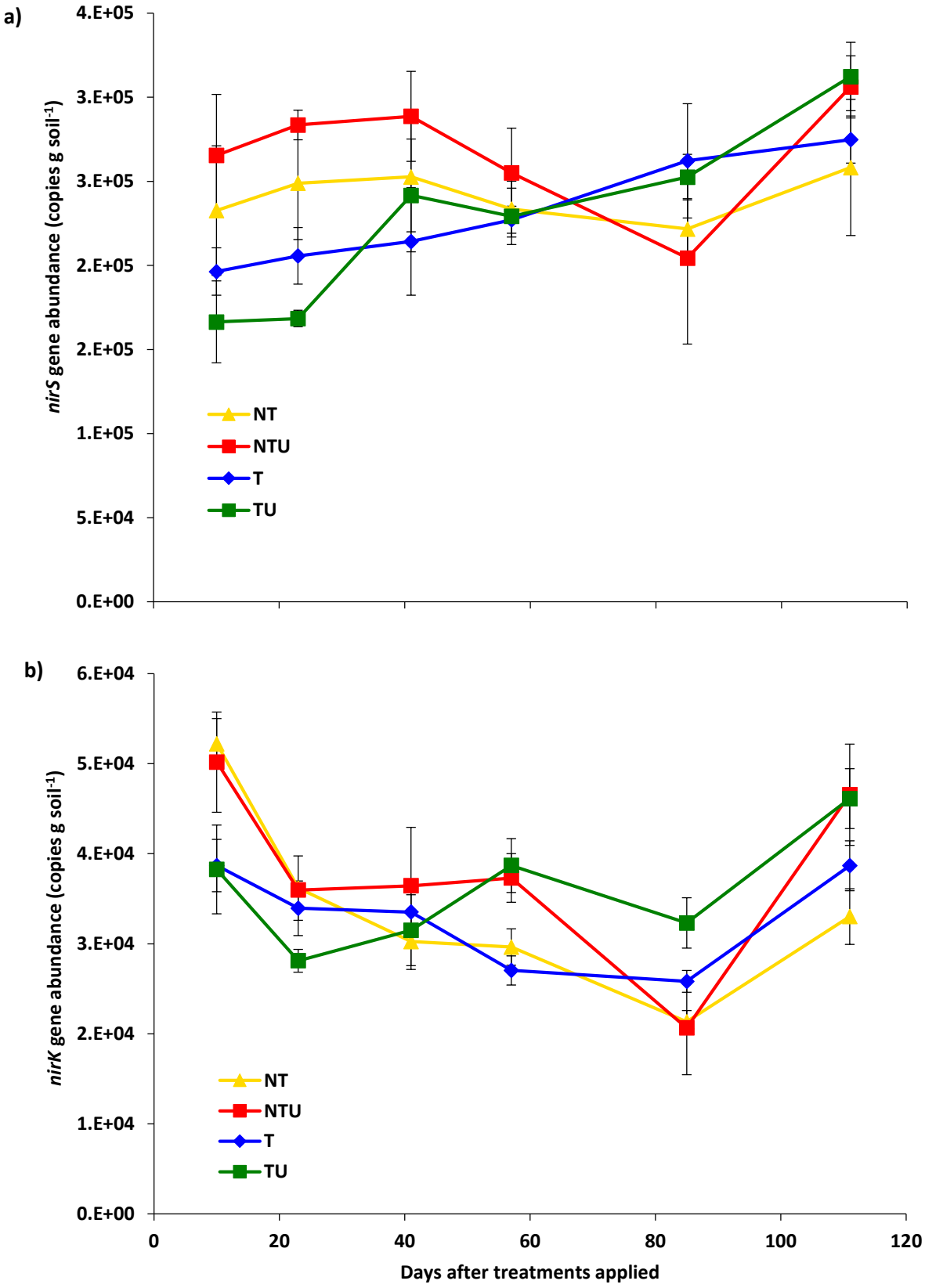

Figure 7.15: **Abundance of denitrifying NO_2^- reductase (a) *nirS*, and (b) *nirK* genes copies in the soil of all treatments. Error bars represent one standard error of the mean.**

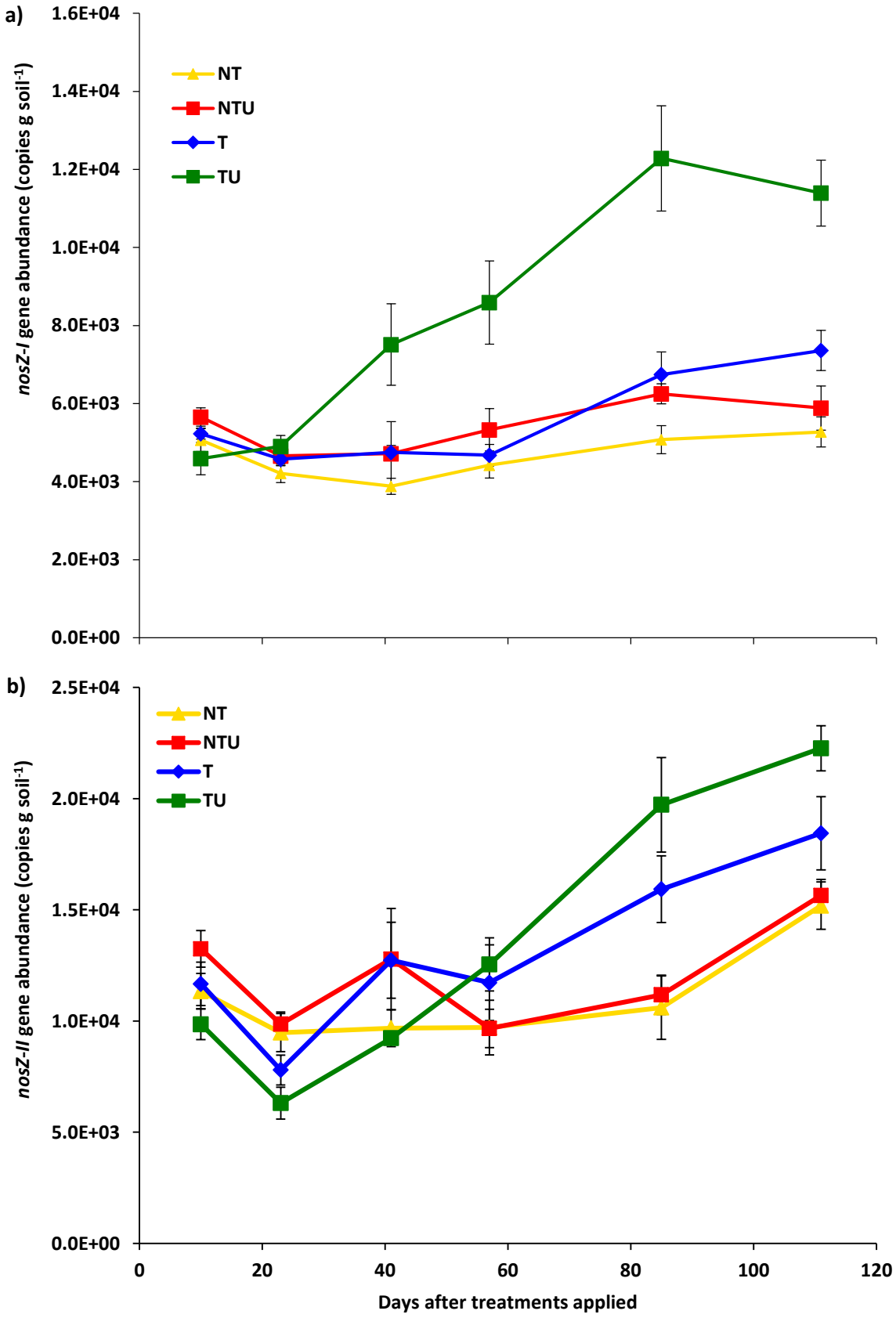

Figure 7.16: **Abundance of denitrifying N_2O reductase *nosZ* (a) clade I, and (b) clade II gene copies the soil of all treatments. Error bars represent one standard error of the mean.**

7.3.5 Physical measurements

Soil Bulk Density

The bulk density of the fine earth fraction of the trampled soil was 1.19 g cm^{-3}, while the bulk density of the fine earth fraction of the non-trampled soil was significantly ($P<0.05$) less at 1.03 g cm^{-3} ($P<0.05$). The soil had, on average, 30% stones by volume. The bulk density of the soil taken from the soil rings was not significantly different between trampled and non-trampled soil until the stones were removed (Table 7.6).

Table 7.6: Bulk density of whole soil, and bulk density of fine earth fraction, with volume of stones greater than 2 mm. Samples taken 13st November, 2013 – 113 days after treatments were applied.

Treatment	Whole soil bulk density (g cm^{-3})	Fine earth bulk density (g cm^{-3})	Stone content (% v/v)
Trampled	1.46	1.19	28
Non-trampled	1.41	1.03	31
LSD# (0.05)	0.14	0.10	-

#Least significant difference ($P < 0.05$).

Soil moisture

The soil moisture content determined gravimetrically when samples were taken from mineral N and microbial assays was very similar between trampled and non-trampled soil. However, when the bulk density of the fine earth fraction of the soil was considered, the volumetric water content of the fine earth fraction of the trampled soil was, on average, higher than that of the non-trampled soil ($P<0.05$) (Figure 7.17).

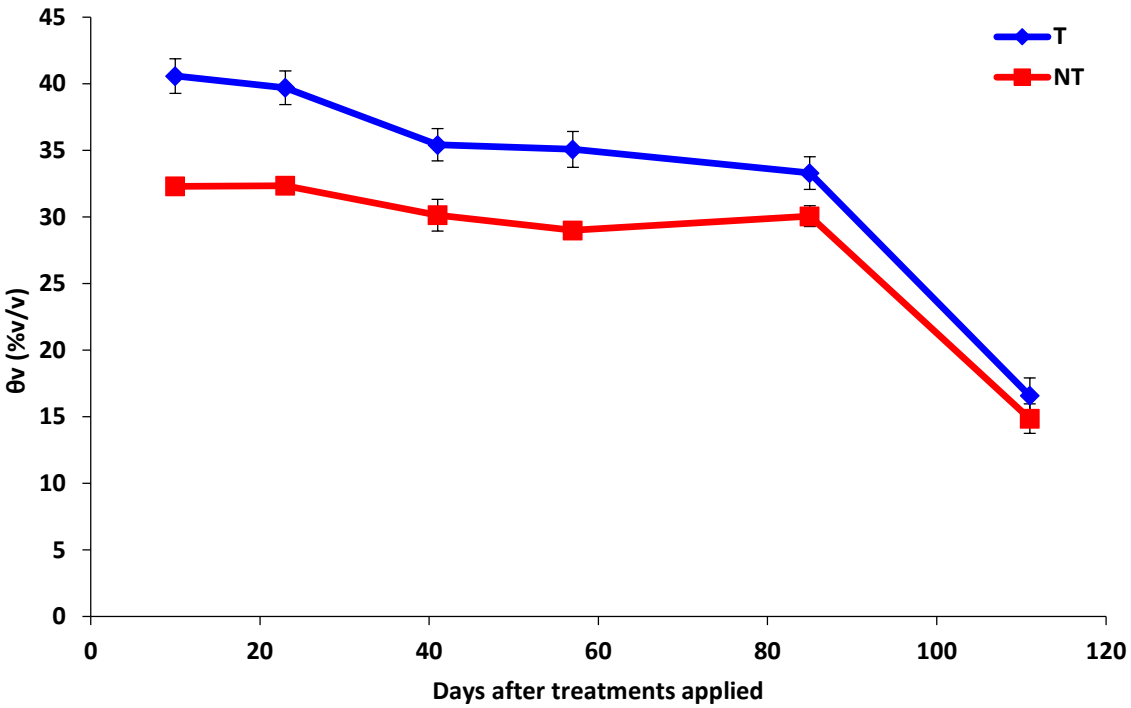

Figure 7.17: **Volumetric soil moisture content in trampled (T) and non-trampled (NT) soil in samples taken for mineral N analysis. Error bars represent one standard error of the mean.**

Air permeability

The pressure transducer used in this experiment had a much larger measurement range than the one described in Chapter Six. Consequently, air permeability (AP) measurements were able to be made and a positive linear correlation was observed between the pressure within the AP chamber and the air flow rate for both trampled and non-trampled soil (R^2=0.99). Simulated animal trampling significantly reduced the AP of the stony Balmoral soil more than 1,000-fold (P<0.01), while dairy cow urine had no significant effect on the AP (Table 7.7). The AP of the non-trampled soil was not significantly different between any of the times it was measured. However, a slight increase (P<0.05) in the AP of the trampled soil was observed 113 days after treatments were applied (Figure 7.18).

Table 7.7: **Log transformed soil air permeability with back transformed (BT) means, 9, 23, and 113 days after treatments were applied.**

Treatment	AP 9 days (m^2)		AP 23 days (m^2)		AP 113 days (m^2)		LSD# (0.05)
	$Log_{10}AP$	BT mean	$Log_{10}AP$	BT mean	$Log_{10}AP$	BT mean	
T	-12.20	7.16×10^{-13}	-12.16	7.50×10^{-13}	-11.79	1.93×10^{-12}	0.35
TU	-12.19	7.86×10^{-13}	-12.37	4.57×10^{-13}	-11.85	1.46×10^{-12}	0.30
NT	-11.13	1.18×10^{-11}	-11.20	7.21×10^{-12}	-11.14	7.44×10^{-12}	0.39
NTU	-11.14	9.33×10^{-12}	-11.15	9.80×10^{-12}	-11.07	1.02×10^{-11}	0.44
LSD# (0.05)	0.44	-	0.34	-	0.30	-	-

#Least significant difference of log transformed means ($P < 0.05$).

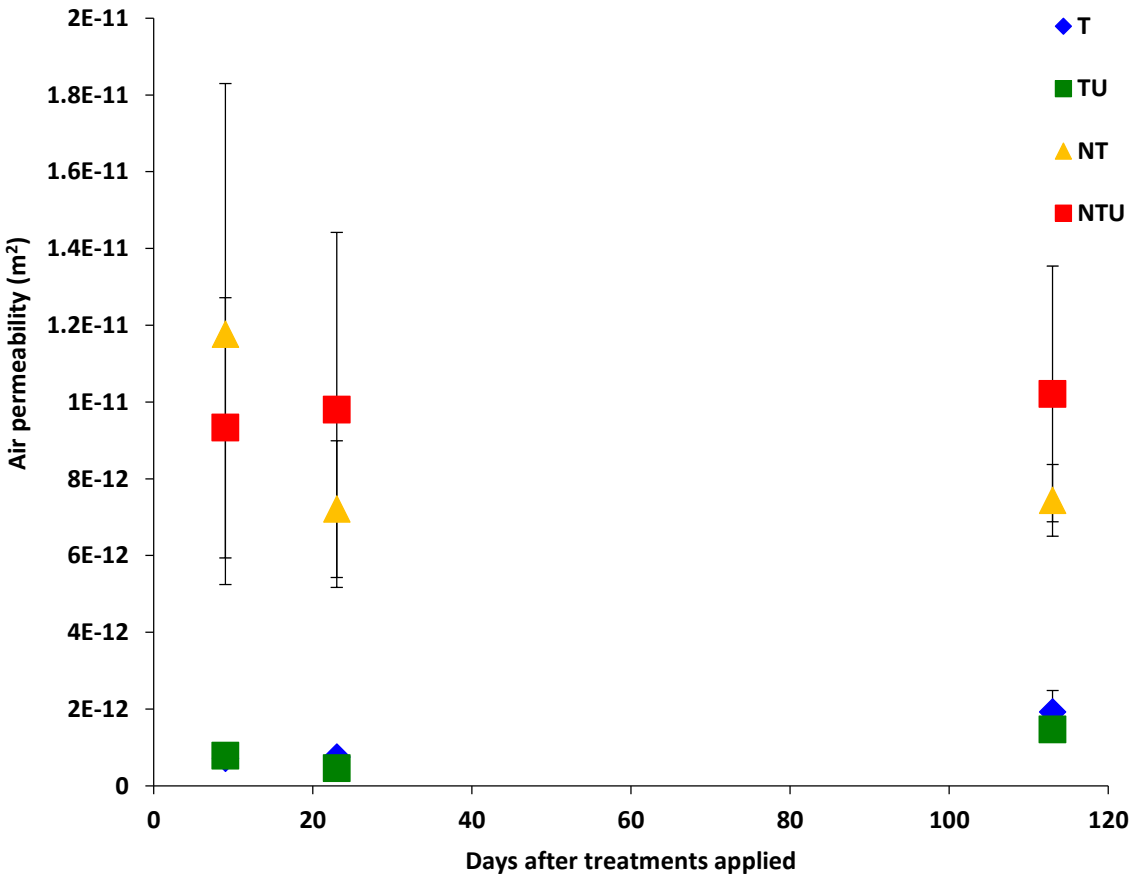

Figure 7.18: **Air permeability of the soil of each treatment. Error bars represent one standard error of the mean.**

7.4 Discussion

Soil compaction and animal trampling have been associated with increased denitrification and accompanying N_2O emission (e.g. Bhandral *et al.* 2007, Thomas *et al.* 2008, Ball *et al.* 2012). However, to date, no authors have linked animal trampling or soil compaction with changes in the size of the microbial denitrifying population in a field study. In the present experiment, copies of denitrifying *nosZ* genes were more abundant in trampled soil than in non-trampled soil (Table 7.5, Figure 7.16a, b), which provides a strong line of evidence to support the hypothesis that animal trampling enhanced denitrification and N_2O emission.

When urine was applied at 400 kg N ha^{-1}, the trampled soil (TU) in the present experiment emitted 6.3 kg N_2O-N ha^{-1}, which compares well with the results in Chapter Six, where the TU soil emitted 7.2 kg N_2O-N ha^{-1}. Similarly, the trampled soil without urine (T) emitted 1.2 kg N_2O-N ha^{-1}, which was not significantly different to the 1.1 kg N_2O-N ha^{-1} emitted by the same treatment in Chapter Six. The proportion of urine-N emitted as N_2O from the trampled soil in the present experiment increased during the period when ^{15}N isotopes of the N_2O were measured. After 48 days, urine-N accounted for 56% of the N_2O emitted from the trampled soil, whereas the maximum proportion of urine-N in the N_2O emitted from the NTU treatment was 5.6%. The proportion of urine-N in the N_2O from the trampled soil may have increased beyond 56% in the latter part of the experiment, but analysis of the proportion of ^{15}N in the N_2O was not assessed beyond the 48th day of the experiment.

Animal trampling significantly increased N_2O emissions in the present experiment when compared with non-trampled soil ($P<0.01$). The non-trampled soil without urine (NT) emitted a total of 0.7 kg N_2O-N ha^{-1}, which was less than the trampled soil (T) ($P<0.05$). The non-trampled soil with urine (NTU) emitted 0.8 kg N_2O-N ha^{-1}, which was almost one eighth of the total N_2O emitted by the trampled soil with urine (TU), which was 6.3 kg N_2O-N ha^{-1} ($P<0.01$). Bhandral *et al.* (2007), reported a three-fold increase in N_2O emission in response to soil compaction, and Menneer *et al.* (2005) observed that soil trampled by dairy cows in the absence of urine emitted more N_2O than non-trampled soil, so the effect of simulated animal trampling on N_2O emissions in the present experiment is in line with that reported previously.

No difference was observed in the average abundance of *nirK* gene copies between trampled and not trampled soil, which is supported by Attard *et al.* (2011) and Le Roux *et al.* (2013), who found *nirK* gene abundance was poorly correlated with potential denitrification. However, Avrahami *et al.* (2002) noted a shift in *nirK* bearing microbial populations with the addition of NH_4^+, which is in agreement with the present experiment, where the application of urine increased the average abundance of *nirK* genes in both trampled and non-trampled soil. Trampling had no significant effect on *nirK* gene abundance, which is similar to Avrahami *et al.* (2002), who did not detect a significant

difference in *nirK* gene abundance in soil from diverse land uses when treated with NO_3^-. Additionally, Yu *et al.* (2014) found *nirS* and *nirK* gene abundance was related to urea addition, but not soil bulk density.

Ruser *et al.* (2006) and Li *et al.* (2014) have demonstrated an increase in denitrification and N_2O emissions following soil compaction using ^{15}N tracer methodology. Hamonts *et al.* (2013) combined ^{15}N tracer methodology with denitrifying microbial DNA analysis in a short term laboratory study but did not observe any correlation between N_2O or N_2 emission and denitrifying gene abundance. The number of NO_2^- reducing *nirS* gene copies in the trampled soil with urine (TU) in this experiment increased by 45% between the 10th and 41st day, and at the same time, the proportion of urine-N derived N_2O emitted increased from 3.3% to about 50%. The correlation between *nirS* gene copies and an increase in the proportion of urine-derived N_2O strongly suggests the urine-N was being denitrified in the trampled soil. In both the trampled soil without urine (T) and the trampled soil with urine (TU) a significant ($P<0.05$) increase in *nirS* gene abundance was observed between the start and end of the experiment (Figure 7.15a). Denitrification will only occur in anaerobic conditions (Zumft 1997), therefore the increase in *nirS* gene copies in the TU soil indicates that the TU soil was sufficiently anaerobic to support the growth of the denitrifying micro-organisms.

As yet, no authors have described changes in denitrifying gene abundance in soil in response to animal trampling or compaction in a field study. In this experiment, more N_2O reducing *nosZ-I* gene copies were present on average in the trampled soil with urine than in the non-trampled soil with urine. Similarly, trampling resulted in more *nosZ-II* gene copies being present in soil with, and without urine. An increase in *nosZ* gene copies indicates more complete denitrification was occurring in the trampled soil, meaning a portion of N was likely to be emitted as N_2 gas. The increase in *nosZ* gene copies in the trampled soil with urine (TU) was accompanied by an increase in the proportion of ^{15}N in the N_2 gas emitted, when compared to the non-trampled soil which received the same ^{15}N labelled urine. The increase in *nosZ* gene copies and increase in proportion of urine-derived N_2 gas from the trampled soil shows that conditions were sufficiently anaerobic to promote the growth of micro-organisms that reduce N_2O to N_2, however it does not imply that all of the N_2O in the soil was reduced to N_2. The difference in the amount of N_2O emitted between the TU and NTU soil represents the N_2O that escaped the soil before complete denitrification occurred.

The increase in abundance of denitrifying gene copies in the trampled soil in the present experiment demonstrates anaerobic conditions were produced, which was reinforced by a corresponding decrease in air permeability (AP) (Table 7.7). The decrease in AP was accompanied by an increase in the bulk density of the fine earth fraction of trampled soil (Table 7.6), which means that the total porosity of the trampled soil was less than that of the non-trampled soil. A well connected pore network is important for gaseous exchange between soil aggregates and the atmosphere, and a

decrease in pore space may have prevented oxygen diffusion into the trampled soil, creating anaerobic conditions required by denitrifying microbes.

In addition to a reduction in gas diffusion, the apparent disruption to the soil pore network would have had implications for drainage of water and urine through the soil. Using AP as an indicator of saturated hydraulic conductivity (Iversen *et al.* 2004, Wells *et al.* 2006), it can be assumed that the higher AP of the non-trampled soil allowed urine to infiltrate deeper into the soil profile when compared to trampled soil. Ten days after the urine was applied, the concentration of NH_4^+ was more than four times higher in the trampled soil than it was in the non-trampled soil, which shows that urine-N was initially held in the upper part of the trampled soil profile (Figure 7.12a). In addition to a greater initial retention of urine-N, the surface ponding observed on trampled soil after rainfall (Figure 6.28) suggests a smaller proportion of the applied N may have leached from the trampled soil profile throughout the duration of the experiment compared to the non-trampled soil. The relatively high average soil NO_3^- concentration in trampled soil with urine (TU) throughout the duration of the experiment strongly suggests less NO_3^- was leached from the trampled soil than non-trampled soil.

Nitrous oxide emissions appear to have largely been emitted during denitrification, as urine had no significant effect on N_2O emissions from the non-trampled soil, even though the concentration of extractable soil NH_4^+ decreased and the concentration of NO_3^- increased, which implies nitrification was occurring. Nitrification also appears to have occurred without producing an elevated N_2O emission in the trampled soil, where the N_2O emission rate had returned to background levels toward the end of the experiment (Figure 7.8) while the soil NO_3^- concentration increased (Figure 7.12b).

The combination of reduced AP, reduced pore space, elevated soil N concentrations, and higher volumetric water content in trampled soil would have provided the necessary conditions for denitrification to occur, giving rise to the elevated N_2O emissions observed. In contrast, the highly permeable, free draining non-trampled soil would have been unlikely to become saturated and anaerobic, which are conditions required for denitrification (Knowles 1982, Zumft 1997, Bateman and Baggs 2005), therefore there was no evidence to suggest an appreciable amount of denitrification had taken place in the non-trampled soil. Consequently, the dissimilarity in total N_2O emitted between the trampled and non-trampled soil implies that the vast majority of N_2O was probably emitted during denitrification, rather than nitrification, in the trampled soil.

7.5 Conclusions

Simulated animal trampling severely affected the physical properties of the stony Balmoral soil used in this experiment. A decrease in the soil pore space in response to simulated trampling was evidenced by an increase in the soil bulk density and a decrease in the soil's air permeability.

The decrease in pore space following trampling affected the drainage properties of the soil, with surface ponding occurring after rainfall and urine application. Ten days after urine was applied, the average NH_4^+ concentration in the top 7.5 cm of soil was more than four times higher in trampled soil than in non-trampled soil, which shows that a large proportion of urine-N was held in the upper portion of the soil profile and not leached. Additionally, the average NO_3^- concentration was much higher in the trampled soil than in the non-trampled soil during the experimental period, implying a smaller portion of urine-N was leached as NO_3^- from the trampled soil than the non-trampled soil.

The reduction in pore space and diminished ability for air to enter the soil following simulated animal trampling created anaerobic conditions which enabled an increase in the size of the denitrifying microbial population. Even without urine addition, more N_2O reducing *nosZ-II* gene copies were found in trampled soil than in the non-trampled soil, indicating denitrifying conditions. The addition of dairy cow urine to trampled soil saw a more than two-fold increase in the abundance of N_2O reducing *nosZ-I* gene copies, and a significant increase in number of the NO_2^- reducing *nirS* gene copies. Together with the increase in the number of *nirS* gene copies, the proportion of urine-N in the N_2O emitted from the trampled soil was ten times higher than in the N_2O emitted from non-trampled soil, indicating urine-derived N was being denitrified through to N_2O. The increase in *nosZ* gene copies was accompanied by an increase in the proportion of ^{15}N labelled urine in the N_2 emitted from the trampled soil. Accordingly, simulated animal trampling significantly increased N_2O emissions, both with, and without urine addition. Therefore, it is highly likely that N_2O emissions increase due to denitrification in a stony soil when dairy cows graze forage crops during the winter months.

Chapter Eight – General Discussion and conclusions

8.1 Overview

A review of previously published work identified an absence of knowledge and understanding about N_2O emissions from forage crops grazed by dairy cows during winter, or ways to mitigate those emissions. The review also highlighted an absence of understanding of the abundance of denitrifying microorganisms in soil in response to animal trampling. The primary hypothesis of this thesis was that animal trampling and urine deposition by dairy cows grazing forage crops in winter would favour denitrifier activity and high rates of N_2O production via denitrification, which could be reduced by the application of DCD and biochar. Four experiments were designed to test this hypothesis – three field experiments, and one laboratory experiment, described in Chapter Four (Year One), Chapter Six (Year Two), Chapter Seven (Year Three) and Chapter Five (Laboratory Experiment).

8.2 Nitrous oxide emissions

In all studies in this thesis, applying dairy cow urine increased the amount of N_2O emitted from the stony Balmoral soil ($P<0.05$). The highest amount of N_2O emitted under field conditions was 13.6 kg N_2O-N ha^{-1}, which occurred in the experiment reported in Chapter Four (Year One), when urine was applied at 700 kg N ha^{-1}. Recent analyses of the N concentration of urine from cows grazing kale has found that the N concentration is lower than that in urine from cows grazing ryegrass/clover pasture (Jenkinson *et al.* 2014). Therefore in the 2nd and 3rd year experiments urine was applied at a lower rate of 400 kg N ha^{-1}. The amount of N_2O emitted from trampled soil in the urine treatments in Year Two was 7.2 kg N_2O-N ha^{-1}, which was not significantly different to that emitted in Year Three, when 6.3 kg N_2O-N ha^{-1} was emitted.

Denitrification in soil following grazing by animals is typically aligned with heavy textured soils with high water contents (e.g. Barton *et al.* 1999, Bateman and Baggs 2005, Ball 2013), which are conditions not generally associated with the stony, free draining Balmoral soil used in this study. For example, Wachendorf *et al.* (2008) reported an EF_3 value (proportion of applied-N emitted as N_2O) of 0.05% when urine was applied to a free draining sandy loam soil at >1,000 kg N ha^{-1}, and a similar value was expected from the free-draining non-trampled Balmoral soil. Accordingly, in Year Three, the EF_3 value for non-trampled soil with urine was 0.03%. However, trampling the stony soil had a significant effect on N_2O emissions in both Year Two ($P<0.05$) and Year Three ($P<0.01$). For the trampled

treatments, EF_3 values ranged from 1.3% to 1.9%, which was considerably higher than from non-trampled soil, but within the range of EF_3 values in a review of 55 field trials by Kelliher *et al.* (2014).

Bhandral *et al.* (2007), and Ball *et al.* (2012) reported increases in emissions of N_2O following urine application and animal trampling of fine-textured soils, which the authors attributed to an increase in denitrification. Similarly, even without urine application, Menneer *et al.* (2005) found higher rates of denitrification in severely trampled fine-textured soil than in non-trampled soil. However, no authors have described the effect of animal trampling on N_2O emissions or denitrification in coarse textured, stony soil. Therefore, the results presented in this thesis confirm that animal trampling of a coarse textured, stony soil, with or without dairy cow urine, increases N_2O emissions.

Dobbie and Smith (2003) correlated EF_3 values from fertilised soil with the soil's water filled pore space (WFPS), and WFPS has been correlated with denitrification (e.g. Davidson *et al.* 1991, Weier *et al.* 1993, Bateman and Baggs 2005). However, in the laboratory experiment (Chapter 5), N_2O emissions were better correlated with the soils volumetric water content (θv) than with the WFPS. In the field trials in this thesis, the trampled soil had a higher θv than the non-trampled soil at all observation points. The increase in θv in trampled soil was a consequence of the reduction in air-filled pores, which was defined by an increase in soil bulk density (BD) following trampling. In Year Two trampling increased the BD of the fine earth fraction of the soil from 0.89 g cm^3 to 1.11 g cm^3 ($P<0.05$), and in Year Three trampling increased the BD of the fine earth fraction of the soil from 1.03 g cm^3 to 1.19 g cm^3 ($P<0.05$).

The increase in WFPS and increase in BD of the soil following trampling was accompanied by a large decrease in the air permeability (AP) ($P<0.01$). These results are in agreement with Ball *et al.* (2008) who also found increases in soil WFPS and BD, and decreases in AP following compaction. Ball (2013) described how soil air permeability divided by air filled porosity could be used as an index of pore continuity. Although there was a small proportional reduction in air filled porosity in Year Two and Year Three when soil was trampled, the orders of magnitude decrease in air permeability indicates a drastic reduction in pore continuity under trampling. A reduction in soil pore continuity following compaction reduces the rate at which O_2 can diffuse into soil, increasing the likelihood of denitrification (Bhandral *et al.* 2007, Ball 2013). Evidence for enhanced denitrification following trampling was observed in the Laboratory Experiment, where NO_3^- appeared to be consumed, and the abundance of denitrifying NO_2^- reductase *nirS* gene copies were more abundant in trampled moist soil than in non-trampled and drier soil. However, a stronger line of evidence for denitrification in trampled soil was found in the Year Three field experiment. The abundance of NO_2^- reductase *nirS* gene copies in trampled soil approximately doubled during the 113 day field experiment, which was accompanied by a linear increase in the proportion of ^{15}N labelled urine-N in the N_2O that was emitted from the trampled soil. In contrast, less than 6% of the N_2O emitted from the non-trampled soil in Year

Three was urine-derived. The increase in abundance of *nirS* gene copies in the trampled soil, together with the high proportion of urine-N in the N_2O, strongly indicates a link to increased denitrification due to trampling.

Further to the increase in *nirS* gene copies, and the reduction of urine-derived NO_2^--N to N_2O, more N_2O reductase *nosZ* gene copies were found in the trampled soil than in the non-trampled soil. A higher abundance of *nosZ* gene copies implies that N_2O was being reduced to N_2, which was confirmed by an increase in the concentration of labelled ^{15}N in the N_2 emitted from the trampled soil, compared with the non-trampled soil. Unlike N_2O, N_2 is only produced by denitrifying organisms, and the increase in the proportion of urine-N in the N_2 emitted from the trampled soil demonstrates that complete denitrification was occurring in the trampled soil.

While increased denitrification following soil compaction has previously been characterised using ^{15}N tracer methodology (e.g. Ruser *et al.* 2006, Li *et al.* 2014), at the time of writing, no other authors had described changes in denitrifying gene abundance in soil in response to animal trampling or compaction in a field study. The work presented in this thesis validates the hypothesis that animal trampling damage and urine deposition by dairy cows grazing forage crops in winter, favours denitrifier activity and high rates of N_2O production via denitrification.

8.3 Nitrous oxide emission mitigation

In Year One, the nitrification inhibitor, DCD, the carbon-rich biochar, and their combination, were trialled to mitigate N_2O emissions. In line with previously published results (e.g. Di and Cameron 2003, de Klein *et al.* 2011, Cameron *et al.* 2014), DCD reduced the amount of N_2O emitted from lysimeters in Year One by 68%, and by up to 44% in Year Two. While DCD successfully reduced N_2O emissions under field conditions, it was even more successful in the Laboratory Experiment, where N_2O emissions were reduced over a 212 day period by up to 91%. In the Laboratory experiment, DCD was effective in reducing N_2O emissions from soil at three different soil water contents, and from trampled and non-trampled soil. When measured in the Laboratory and Year Two experiments, soil with urine and DCD had a higher average NH_4^+-N concentration, and a lower average NO_3^--N concentration, than soil with urine and no DCD. DCD also delayed the observed peak NO_3^--N concentration in both studies, which demonstrated that the nitrification inhibitor was effective in delaying nitrification in both field and laboratory conditions.

It was hypothesised that the increase in soil NH_4^+-N concentration following DCD application (Di and Cameron 2008, Smith *et al.* 2008b, Moir *et al.* 2012) may have a complementary effect with the possible increase in soil cation exchange capacity brought about by biochar incorporation

(Lehmann 2007a, Atkinson *et al.* 2010, Ding *et al.* 2010). The possible complimentary effect between DCD and biochar may have reduced the substrate available for denitrification, and subsequently reduced N_2O emissions below that of either biochar-only or DCD-only treatments. As yet, no other authors have described the effect of the combination of DCD and biochar on N_2O emissions from soil. However, the use of biochar at 5 t ha^{-1} (1% of soil dry weight) in Year One, whether alone or in combination with DCD, had no significant effect on N_2O emissions. Similarly, Suddick and Six (2013), and Angst *et al.* (2014) found biochar had no effect on N_2O emissions when applied to N-rich soil at rates between 5 and 18.8 t ha^{-1}. On the contrary, in a review, Cayuela *et al.* (2014) reported that biochar applied at a rate of 1-2% of soil mass would be likely to achieve an average 27% reduction in N_2O emissions. Since the beginning of this research project in 2011 to the time of writing, the number of studies identified by the keyword 'biochar' in the Web of Science® database has increased from about 250 to about 5,500. Yet despite the substantial amount of new biochar related research, in recent reviews, Clough *et al.* (2013a), and Cayuela *et al.* (2014) found the key mechanisms by which biochar affects N_2O emissions from soil are largely controversial and still poorly understood.

8.4 Final conclusions

- Trampling and urine deposition created anaerobic conditions which favoured denitrifier activity, and increased N_2O emissions.
- Dairy cow urine significantly ($P<0.05$) increased the amount of N_2O emitted from trampled soil.
- The abundance of denitrifying *nirS* and *nosZ* gene copies in a coarse textured, stony soil increased following trampling.
- Trampling increased soil volumetric water content.
- Trampling significantly ($P<0.05$) increased the bulk density of the fine earth fraction of the stony Balmoral soil.
- Trampling severely decreased the soil air permeability.
- In field conditions, DCD reduced N_2O emissions from urine amended, trampled soil by 44% to 68%.
- DCD was more effective in reducing N_2O emissions in trampled soil than in non-trampled soil.
- Biochar incorporated into soil at 5 t ha^{-1} had no significant effect on N_2O emissions.
- Combining DCD and biochar was no more effective than using DCD alone to mitigate N_2O emissions.
- In laboratory conditions, DCD reduced N_2O emissions from urine amended soil by 77% to 91%, and was most effective in trampled, moist soil.
- In laboratory conditions, N_2O emissions were more closely correlated with volumetric water content than water filled pore space.

8.5 Suggestions for further research

The objectives of this research project were to quantify N_2O emissions from urine, and non-urine affected soil, and to investigate the effects of animal trampling on soil physical conditions, and soil microbial populations in a winter dairy forage grazing system. However, there are several opportunities for further research.

Quantification of N_2 emissions

An increase in the amount of urine-derived N was detected in the N_2 emitted from trampled soil, but the amount of N_2 emitted was not quantified. Quantification of N_2 emissions will help create a more complete N budget for the winter dairy forage grazing system, and improve understanding of the scale of denitrification occurring.

Field-scale N_2O emissions

Important factors to consider when calculating field-scale N_2O emissions include; the location of urine deposition; the volume of urine excreted; the number or urine events; the urine infiltration depth; the area covered by urine; and, the N concentration of the urine. However, these variables were outside the scope of research in this thesis and were not quantified. When quantifying N_2O emissions at the field-scale, it is also necessary to consider the amount of NO_3^--N leached, as the IPCC takes into account N_2O emitted from NO_3^--N leached to groundwater, streams, rivers, and oceans etc. (IPCC 2013).

When observing dairy cows grazing kale at the Lincoln University Ashley Dene farm, the permeability and micro-topography of the soil had a large effect on the area of soil covered by a single cow urine event. Field observations showed urine deposited onto smooth, impermeable soil, covered a larger area than urine deposited onto rough, wet soil. Moreover, when cows urinated on soil that did not appear to have suffered trampling damage, the urine was absorbed almost immediately, much like what was seen when urine was applied to the non-trampled soil in Year Two and Year Three.

There is a limited amount of research describing animal urine distribution in grazed pasture (e.g. Betteridge *et al.* 2010, Dennis *et al.* 2011, Moir *et al.* 2011), but a complete absence of research into urine distribution in forage grazing. A better understanding of urine patch distribution in forage grazing systems will enable more accurate field-scale N_2O emission budgets to be calculated.

Soil moisture content at time of grazing

Field observations also showed that the soil moisture status at the time of grazing had a dramatic effect on the degree of trampling damage induced by the dairy cows (Figure 8.1). The effect of soil moisture content at the time of trampling on the degree of soil damage could be investigated further.

Figure 8.1: Considerably more trampling damage occurred when soil was grazed in wet conditions rather than in drier conditions. The soil at right was grazed during wet conditions, and the soil at left grazed the following day. The obvious line through the middle of the photograph signifies where a fence was placed to restrict cow movement.

Restricted grazing

A standoff pad constructed beside the forage crop may be used to limit trampling damage to the soil, which may reduce net N_2O emissions. Jenkinson *et al.* (2014) observed that cows grazing kale had eaten about 75% of their daily feed intake within 6 hours, and possibly, the proportion of feed eaten could be enhanced by restricted grazing, as Oudshoorn *et al.* (2008) found that the rate dairy cows consumed feed increased as grazing time was reduced. The stony Balmoral soil did not appear to have suffered trampling damage after six hours of grazing by dairy cows, although the degree of soil

damage in response to grazing time was not investigated in this thesis. Promisingly, a standoff pad was found to emit only 0.01% of applied urine-N as N_2O (Luo and Saggar 2008), and the combination of a standoff pad plus DCD was found to reduce farm-scale N_2O emissions between 43 and 79% (Luo *et al.* 2013b). The possibility of using a standoff pad in conjunction with winter forage grazing to reduce N_2O emissions has not yet been tested.

Other soil inputs

Cows that graze kale are given further feed supplements to provide a balanced diet. Straw is a common supplement, which is often placed directly on previously grazed soil for cows to graze ad-lib. However, not all of the straw is eaten, and a portion of the straw can become trampled into the soil (Figure 8.2). Incorporation of straw residue has been shown to induce changes in soil physical properties (Ball *et al.* 1990), which could help prevent anaerobic conditions from developing. Accordingly, N_2O emissions were found to increase with distance from straw residue that had become incorporated into grazed soil (Ball *et al.* 2000b). Trampled soil where straw residue had been incorporated could emit less N_2O than what was seen in the field experiments of this thesis, and further investigation is warranted.

Figure 8.2: **Not all of the straw fed to supplement the kale (left) is eaten by the cows, and some becomes trampled into the soil (right).**

Observations showed that a large amount of animal dung was deposited on the soil in winter grazed forage systems. Particularly in wet conditions, the movement of the dairy cows was seen to be working the dung and urine into the soil. Although N_2O emissions associated with animal dung have been found to be considerably less than emissions associated with animal urine (Luo *et al.* 2013a), dung could provide a source of carbon for denitrification of urine-N (Saggar *et al.* 2013), which could change the N_2O and/or N_2 emission rates from those seen in the experiments in this thesis.

Acknowledgements

I seem to have acquired a substantial amount of grey hair during the production of this thesis. Even so, I am confident that I would be considerably greyer had I not had the help and guidance from several important people.

I have thoroughly enjoyed working with my supervisors Professor Hong Di and Professor Keith Cameron in the Centre for Environmental Research, and so I thank them for giving me the opportunity to work with them. I thank them for their encouragement and guidance throughout this project, and for their generous support on the path beyond my studies.

A big thank you also to Dr Barbara Brown for proof reading my work, and all her other assistance. I have received excellent support from the technical staff at Lincoln, particularly Carole Barlow, Jeneth Hendry, Shelagh Bassett, Andriy Podolyan, Steve Moore, Roger Atkinson, Trevor Hendry, Chris Abraham, Neil Smith, Jie Lei, Barry Anderson, and Manjula Premarante, and to those people I say thank you. A further thank you to Dr Bruce Ball for his technical assistance.

I would also like to acknowledge Aimee Robinson and Brendon Malcolm, whose great conversations when assisting me with field work made my task much more enjoyable. I would also like to thank Anne-Maree Hill for her assistance throughout the project.

A big thank you to Julie and Evan for all the good things parents do, and to Justin and Leah who made it possible for me to move to Christchurch to begin this project.

A special thank you to Amal Torky for all things administrative, and for ensuring that I got much more than a qualification at Lincoln by putting me in an office with Eva.

And finally, thank you Eva for joining me on this journey, I am sure there will be many more to come.

References

Aland, A., L. Lidfors and I. Ekesbo (2002). Diurnal distribution of dairy cow defecation and urination. *Applied Animal Behaviour Science* **78**(1): 43-54.

Ameloot, N., S. Neve, K. Jegajeevagan, G. Yildiz, D. Buchan, Y. N. Funkuin, W. Prins, L. Bouckaert and S. Sleutel (2013). Short-term CO_2 and N_2O emissions and microbial properties of biochar amended sandy loam soils. *Soil Biology & Biochemistry* **57**: 401-410.

Angst, T. E., J. Six, D. S. Reay and S. P. Sohi (2014). Impact of pine chip biochar on trace greenhouse gas emissions and soil nutrient dynamics in an annual ryegrass system in California. *Agriculture Ecosystems & Environment* **191**: 17-26.

Arthur, E., P. Schjonning, P. Moldrup, M. Tuller and L. W. de Jonge (2013). Density and permeability of a loess soil: Long-term organic matter effect and the response to compressive stress. *Geoderma* **193**: 236-245.

Atkinson, C. J., J. D. Fitzgerald and N. A. Hipps (2010). Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review. *Plant and Soil* **337**(1-2): 1-18.

Attard, E., S. Recous, A. Chabbi, C. De Berranger, N. Guillaumaud, J. Labreuche, L. Philippot, B. Schmid and X. Le Roux (2011). Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. *Global Change Biology* **17**(5): 1975-1989.

Avrahami, S., R. Conrad and G. Braker (2002). Effect of Soil Ammonium Concentration on N_2O Release and on the Community Structure of Ammonia Oxidizers and Denitrifiers. *Applied and environmental microbiology* **68**(11): 5685-5692.

Babic, K. H., K. Schauss, B. Hai, S. Sikora, S. Redzepovic, V. Radl and M. Schloter (2008). Influence of different *Sinorhizobium meliloti* inocula on abundance of genes involved in nitrogen transformations in the rhizosphere of alfalfa (Medicago sativa L.). *Environmental microbiology* **10**(11): 2922-2930.

Baggs, E. M. (2008). A review of stable isotope techniques for N_2O source partitioning in soils: recent progress, remaining challenges and future considerations. *Rapid Communications in Mass Spectrometry* **22**(11): 1664-1672.

Ball, B. C. (2013). Soil structure and greenhouse gas emissions: a synthesis of 20 years of experimentation. *European Journal of Soil Science* **64**(3): 357-373.

Ball, B. C., D. C. Bickerton and E. A. G. Robertson (1990). Straw incorporation and tillage for winter barley - soil structural effects. *Soil & Tillage Research* **15**(4): 309-327.

Ball, B. C., K. C. Cameron, H. J. Di and S. Moore (2012). Effects of trampling of a wet dairy pasture soil on soil porosity and on mitigation of nitrous oxide emissions by a nitrification inhibitor, dicyandiamide. *Soil Use and Management* **28**(2): 194-201.

Ball, B. C., D. J. Campbell and E. A. Hunter (2000a). Soil compactibility in relation to physical and organic properties at 156 sites in UK. *Soil & Tillage Research* **57**(1-2): 83-91.

Ball, B. C., I. Crichton and G. W. Horgan (2008). Dynamics of upward and downward N_2O and CO_2 fluxes in ploughed or no-tilled soils in relation to water-filled pore space, compaction and crop presence. *Soil & Tillage Research* **101**(1-2): 20-30.

Ball, B. C., B. S. Griffiths, C. F. E. Topp, R. Wheatley, R. L. Walker, R. M. Rees, C. A. Watson, H. Gordon, P. D. Hallett, B. M. McKenzie and I. M. Nevison (2014). Seasonal nitrous oxide emissions from field soils under reduced tillage, compost application or organic farming. *Agriculture Ecosystems & Environment* **189**: 171-180.

Ball, B. C., W. Harris and J. R. Burford (1981). A laboratory method to measure gas diffusion and flow in soil and other porous materials. *Journal of Soil Science* **32**(3): 323-&.

Ball, B. C., G. W. Horgan and J. P. Parker (2000b). Short-range spatial variation of nitrous oxide fluxes in relation to compaction and straw residues. *European Journal of Soil Science* **51**(4): 607-616.

Ball, B. C., J. P. Parker and A. Scott (1999a). Soil and residue management effects on cropping conditions and nitrous oxide fluxes under controlled traffic in Scotland. 2). Nitrous oxide, soil N status and weather. *Soil & Tillage Research* **52**(3-4): 191-201.

Ball, B. C. and P. Schjonning (2002). Air Permeability. *Methods of Soil Analysis, Part 4: Physical Methods*. J. H. Dane and G. C. Topp. Madison, WI, Soil Science Society of America**:** 1141-1158.

Ball, B. C., A. Scott and J. P. Parker (1999b). Field N_2O, CO_2 and CH_4 fluxes in relation to tillage, compaction and soil quality in Scotland. *Soil & Tillage Research* **53**(1): 29-39.

Barrett, M., M. Jahangir, C. Lee, C. Smith, N. Bhreathnach, G. Collins, K. Richards and V. O'Flaherty (2013). Abundance of denitrification genes under different peizometer depths in four Irish agricultural groundwater sites. *Environmental Science and Pollution Research* **20**(9): 6646-6657.

Barta, J., T. Melichova, D. Vanek, T. Picek and H. Santruckova (2010). Effect of pH and dissolved organic matter on the abundance of *nirK* and *nirS* denitrifiers in spruce forest soil. *Biogeochemistry* **101**(1-3): 123-132.

Barton, L., C. D. A. McLay, L. A. Schipper and C. T. Smith (1999). Annual denitrification rates in agricultural and forest soils: a review. *Australian Journal of Soil Research* **37**(6): 1073-1093.

Bartossek, R., G. W. Nicol, A. Lanzen, H. P. Klenk and C. Schleper (2010). Homologues of nitrite reductases in ammonia-oxidizing archaea: diversity and genomic context. *Environmental microbiology* **12**(4): 1075-1088.

Bateman, E. J. and E. M. Baggs (2005). Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biology and Fertility of Soils* **41**(6): 379-388.

Batey, T. (2009). Soil compaction and soil management - a review. *Soil Use and Management* **25**(4): 335-345.

Beare, M. H., E. G. Gregorich and P. St-Georges (2009). Compaction effects on CO_2 and N_2O production during drying and rewetting of soil. *Soil Biology & Biochemistry* **41**(3): 611-621.

Bedard, C. and R. Knowles (1989). Physiology, Biochemistry, and Specific Inhibitors of CH_4, NH_4^+, and CO Oxidation by Methanotrophs and Nitrifiers. *Microbiological Reviews* **53**(1): 68-84.

Betteridge, K., C. Hoogendoorn, D. Costall, M. Carter and W. Griffiths (2010). Sensors for detecting and logging spatial distribution of urine patches of grazing female sheep and cattle. *Computers and Electronics in Agriculture* **73**(1): 66-73.

Betteridge, K., A. D. Mackay, T. G. Shepherd, D. J. Barker, P. J. Budding, B. P. Devantier and D. A. Costall (1999). Effect of cattle and sheep treading on surface configuration of a sedimentary hill soil. *Australian Journal of Soil Research* **37**(4): 743-760.

Bhandral, R., S. Saggar, N. S. Bolan and M. J. Hedley (2007). Transformation of nitrogen and nitrous oxide emission from grassland soils as affected by compaction. *Soil & Tillage Research* **94**(2): 482-492.

Bilotta, G. S., R. E. Brazier and P. M. Haygarth (2007). The impacts of grazing animals on the quality of soils, vegetation, and surface waters in intensively managed grasslands. *Advances in Agronomy, Vol 94*. D. L. Sparks. San Diego, Elsevier Academic Press Inc. **94:** 237-280.

Blackmer, A. M. and J. M. Bremner (1978). Inhibitory effect of nitrate on reduction of N_2O to N_2 by soil microorganisms. *Soil Biology & Biochemistry* **10**(3): 187-191.

Blackmer, A. M., J. M. Bremner and E. L. Schmidt (1980). Production of nitrous oxide by ammonia oxidising chemautotrophic microorganisms in soil. *Applied and environmental microbiology* **40**(6): 1060-1066.

Booth, M. S., J. M. Stark and E. Rastetter (2005). Controls on nitrogen cycling in terrestrial ecosystems: A synthetic analysis of literature data. *Ecological Monographs* **75**(2): 139-157.

Bothe, H., G. Jost, M. Schloter, B. B. Ward and K. P. Witzel (2000). Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS microbiology reviews* **24**(5): 673-690.

Bouwman, A. F., Ed. (1990). *Soils and the greenhouse effect*, John Wiley and Sons Ltd.

Bouwman, A. F. (1998). Environmental science - Nitrogen oxides and tropical agriculture. *Nature* **392**(6679): 866-867.

Braker, G. and R. Conrad (2011). Diversity, structure, and size of N_2O-producing microbial communities in soils-what matters for their functioning? *Advances in applied microbiology* **75**: 33-70.

Braker, G., A. Fesefeldt and K. P. Witzel (1998). Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and environmental microbiology* **64**(10): 3769-3775.

Braker, G., J. Z. Zhou, L. Y. Wu, A. H. Devol and J. M. Tiedje (2000). Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific northwest marine sediment communities. *Applied and environmental microbiology* **66**(5): 2096-2104.

Brankatschk, R., S. Towe, K. Kleineidam, M. Schloter and J. Zeyer (2011). Abundances and potential activities of nitrogen cycling microbial communities along a chronosequence of a glacier forefield. *The ISME journal* **5**(6): 1025-1037.

Bremner, J. (1997). Sources of nitrous oxide in soils. *Nutrient Cycling in Agroecosystems* **49**(1): 7-16.

Bruun, E. W., D. Muller-Stover, P. Ambus and H. Hauggaard-Nielsen (2011). Application of biochar to soil and N_2O emissions: potential effects of blending fast-pyrolysis biochar with anaerobically digested slurry. *European Journal of Soil Science* **62**(4): 581-589.

Bussink, D. W. and O. Oenema (1998). Ammonia volatilization from dairy farming systems in temperate areas: a review. *Nutrient Cycling in Agroecosystems* **51**(1): 19-33.

Cameron, K. C. (1992). Nitrogen in Soil. *Encyclopedia of Earth System Science.* London, UK., Academic Press. **3:** 307-317.

Cameron, K. C., H. J. Di and J. L. Moir (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology* **162**(2): 145-173.

Cameron, K. C., H. J. Di and J. L. Moir (2014). Dicyandiamide (DCD) effect on nitrous oxide emissions, nitrate leaching and pasture yield in Canterbury, New Zealand. *New Zealand Journal of Agricultural Research*: 1-20.

Cameron, K. C., N. P. Smith, C. D. A. McLay, P. M. Fraser, R. J. McPherson, D. F. Harrison and P. Harbottle (1992). Lysimeters without ede flow - an improved design and sampling procedure. *Soil Science Society of America Journal* **56**(5): 1625-1628.

Carran, R. A., P. W. Theobald and J. P. Evans (1995). Emission of nitrous oxide from some grazed pasture soils in New Zealand. *Australian Journal of Soil Research* **33**(2): 341-352.

Carrick, S., D. Palmer, T. Webb, J. Scott and L. Lilburne (2013). Stony soils are a major challenge for nutrient management under irrigation development. *Accurate and efficient use of nutrients on farms.* (Eds L.D. Currie and C L. Christensen). Occasional Report No. 26. Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand. 8 pages..

Carter, M. S. (2007). Contribution of nitrification and denitrification to N_2O emissions from urine patches. *Soil Biology and Biochemistry* **39**(8): 2091-2102.

Case, S. D. C., N. P. McNamara, D. S. Reay and J. Whitaker (2012). The effect of biochar addition on N_2O and CO_2 emissions from a sandy loam soil - The role of soil aeration. *Soil Biology & Biochemistry* **51**: 125-134.

Castro-Gonzalez, M., G. Braker, L. Farias and O. Ulloa (2005). Communities of *nirS*-type denitrifiers in the water column of the oxygen minimum zone in the eastern South Pacific. *Environmental microbiology* **7**(9): 1298-1306.

Cayuela, M. L., M. A. Sanchez-Monedero, A. Roig, K. Hanley, A. Enders and J. Lehmann (2013). Biochar and denitrification in soils: when, how much and why does biochar reduce N_2O emissions? *Scientific Reports* **3**: 7.

Cayuela, M. L., L. van Zwieten, B. P. Singh, S. Jeffery, A. Roig and M. A. Sanchez-Monedero (2014). Biochar's role in mitigating soil nitrous oxide emissions: A review and meta-analysis. *Agriculture Ecosystems & Environment* **191**: 5-16.

Chan, K. Y., L. Van Zwieten, I. Meszaros, A. Downie and S. Joseph (2007). Agronomic values of greenwaste biochar as a soil amendment. *Australian Journal of Soil Research* **45**(8): 629-634.

Chapuis-Lardy, L., N. Wrage, A. Metay, J.-L. Chotte and M. Bernoux (2007). Soils, a sink for N_2O? A review. *Global Change Biology* **13**(1): 1-17.

Chen, Z., X. Luo, R. Hu, M. Wu, J. Wu and W. Wei (2010). Impact of long-term fertilization on the composition of denitrifier communities based on nitrite reductase analyses in a paddy soil. *Microbial ecology* **60**(4): 850-861.

Chief, K., T. P. A. Ferre and A. C. Hinnell (2008a). The effects of anisotropy on in-situ air permeability measurements. *Vadose Zone Journal* **7**(3): 941-947.

Chief, K., T. P. A. Ferre and B. Nijssen (2008b). Correlation between Air Permeability and Saturated Hydraulic Conductivity: Unburned and Burned Soils. *Soil Science Society of America Journal* **72**(6): 1501-1509.

Chroňáková, A., V. Radl, J. Čuhel, M. Šimek, D. Elhottová, M. Engel and M. Schloter (2009). Overwintering management on upland pasture causes shifts in an abundance of denitrifying microbial communities, their activity and N_2O-reducing ability. *Soil Biology and Biochemistry* **41**(6): 1132-1138.

Clough, T. J., J. E. Bertram, J. L. Ray, L. M. Condron, M. O'Callaghan, R. R. Sherlock and N. S. Wells (2010). Unweathered Wood Biochar Impact on Nitrous Oxide Emissions from a Bovine-Urine-Amended Pasture Soil. *Soil Science Society of America Journal* **74**(3): 852-860.

Clough, T. J. and L. M. Condron (2010). Biochar and the Nitrogen Cycle: Introduction. *Journal of environmental quality* **39**(4): 1218-1223.

Clough, T. J., L. M. Condron, C. Kammann and C. Müller (2013a). A review of biochar and soil nitrogen dynamics. *Agronomy* **3**(2): 275-293.

Clough, T. J., H. J. Di, K. C. Cameron, R. R. Sherlock, A. K. Metherell, H. Clark and G. Rys (2007). Accounting for the utilization of a N_2O mitigation tool in the IPCC inventory methodology for agricultural soils. *Nutrient Cycling in Agroecosystems* **78**(1): 1-14.

Clough, T. J., S. F. Ledgard, M. S. Sprosen and M. J. Kear (1998). Fate of ^{15}N labelled urine on four soil types. *Plant and Soil* **199**(2): 195-203.

Clough, T. J., C. Muller and R. J. Laughlin (2013b). Using stable isotopes to follow excreta N dynamics and N_2O emissions in animal production systems. *Animal* **7** (2): 418-426.

Conrad, R. (1996). Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiological Reviews* **60**(4): 609-640.

Coppola, A., G. Dragonetti, A. Comegna, N. Lamaddalena, B. Caushi, M. A. Haikal and A. Basile (2013). Measuring and modeling water content in stony soils. *Soil & Tillage Research* **128**: 9-22.

Correa-Galeote, D., G. Tortosa and E. J. Bedmar (2013). Determination of denitrification genes abundance in environmental samples. *Metagenimics* **2**: 1-14.

Cousin, I., B. Nicoullaud, M. Tetegan, A. C. R. de Forges, D. Arrouays and A. Bouthier (2014). Estimating the Available Water Content of highly heterogeneous soils including stony soils at the regional scale. *GlobalSoilMap: Basis of the global spatial soil information system*, 221.

Dalal, R. C., W. J. Wang, G. P. Robertson and W. J. Parton (2003). Nitrous oxide emission from Australian agricultural lands and mitigation options: a review. *Australian Journal of Soil Research* **41**(2): 165-195.

Dandie, C. E., D. L. Burton, B. J. Zebarth, J. T. Trevors and C. Goyer (2007). Analysis of denitrification genes and comparison of *nosZ*, *cnorB* and 16S rDNA from culturable denitrifying bacteria in potato cropping systems. *Systematic and applied microbiology* **30**(2): 128-138.

Dandie, C. E., S. Wertz, C. L. Leclair, C. Goyer, D. L. Burton, C. L. Patten, B. J. Zebarth and J. T. Trevors (2011). Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. *FEMS microbiology ecology* **77**(1): 69-82.

Davidson, E. A. (1993). *Soil water content and the ratio of nitrous oxide to nitric oxide emitted from soil*. London, Chapman & Hall.

Davidson, E. A. (2009). The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nature Geoscience* **2**(9): 659-662.

Davidson, E. A., S. C. Hart, C. A. Shanks and M. K. Firestone (1991). Measuring gross nitrogen mineralisation, immobilisation, and nitrification by ^{15}N isotopic pool dilution in intact soil cores. *Journal of Soil Science* **42**(3): 335-349.

De Boodt, M. F. and D. Kirkham (1953). Anisotropy and measurement of air permeability of soil clods. *Soil Science* **76**: 127-133.

de Klein, C. A. M., L. Barton, R. R. Sherlock, Z. Li and R. P. Littlejohn (2003). Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. *Australian Journal of Soil Research* **41**(3): 381-399.

de Klein, C. A. M., K. C. Cameron, H. J. Di, G. Rys, R. M. Monaghan and R. R. Sherlock (2011). Repeated annual use of the nitrification inhibitor dicyandiamide (DCD) does not alter its effectiveness in reducing N_2O emissions from cow urine. *Animal Feed Science and Technology* **166-167**: 480-491.

de Klein, C. A. M. and R. J. Eckard (2008). Targeted technologies for nitrous oxide abatement from animal agriculture. *Australian Journal of Experimental Agriculture* **48**(1-2): 14-20.

de Klein, C. A. M. and S. F. Ledgard (2001). An analysis of environmental and economic implications of nil and restricted grazing systems designed to reduce nitrate leaching from New Zealand dairy farms. I. Nitrogen losses. *New Zealand Journal of Agricultural Research* **44**(2-3): 201-215.

de Klein, C. A. M. and S. F. Ledgard (2005). Nitrous oxide emissions from New Zealand agriculture - key sources and mitigation strategies. *Nutrient Cycling in Agroecosystems* **72**(1): 77-85.

de Klein, C. A. M., C. Pinares-Patino and G. C. Waghorn (2008). *Greenhouse gas emissions*. *Environmental Impacts of Pasture-based Farming*. R.W. Mc Dowell (eds). Wallingford UK, CAB International. 1-32.

de Klein, C. A. M., R. R. Sherlock, K. C. Cameron and T. J. van der Weerden (2001). Nitrous oxide emissions from agricultural soils in New Zealand - a review of current knowledge and directions for future research. *Journal of the Royal Society of New Zealand* **31**(3): 543-574.

de Klein, C. A. M. and R. S. P. van Logtestijn (1994). Denitrification and N_2O Emission From Urine-Affected Grassland Soil. *Plant and Soil* **163**(2): 235-241.

De Klein, C. A. M. and R. S. P. Van Logtestijn (1996). Denitrification in grassland soils in The Netherlands in relation to irrigation, N-application rate, soil water content and soil temperature. *Soil Biology and Biochemistry* **28**(2): 231-237.

Dennis, S. J., J. L. Moir, K. C. Cameron, H. J. Di, D. Hennessy and K. G. Richards (2011). Urine patch distribution under dairy grazing at three stocking rates in Ireland. *Irish Journal of Agricultural and Food Research* **50**(2): 149-160.

Di, H. J. and K. C. Cameron (2002). The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. *Soil Use and Management* **18**: 395-403.

Di, H. J. and K. C. Cameron (2003). Mitigation of nitrous oxide emissions in spray irrigated grazed grassland by treating the soil with dicyandiamide, a nitrification inhibitor. *Soil Use and Management* **19**: 284-290.

Di, H. J. and K. C. Cameron (2004a). Effects of temperature and application rate of a nitrification inhibitor, dicyandiamide (DCD), on nitrification rate and microbial biomass in a grazed pasture soil. *Australian Journal of Soil Research* **42**(8): 927.

Di, H. J. and K. C. Cameron (2004b). Effects of the nitrifcation inhibitor dicyandiamide on potassium, magnesium and calcium leaching in grazed grassland. *Soil Use and Management* **20**: 2-7.

Di, H. J. and K. C. Cameron (2005). Nitrous oxide emissions from two dairy pasture soils as affected by different rates of a fine particle suspension nitrification inhibitor, dicyandiamide. *Biology and Fertility of Soils* **42**(6): 472-480.

Di, H. J. and K. C. Cameron (2007). Nitrate leaching losses and pasture yields as affected by different rates of animal urine nitrogen returns and application of a nitrification inhibitor—a lysimeter study. *Nutrient Cycling in Agroecosystems* **79**(3): 281-290.

Di, H. J. and K. C. Cameron (2008). Sources of nitrous oxide from ^{15}N-labelled animal urine and urea fertiliser with and without a nitrification inhibitor, dicyandiamide (DCD). *Australian Journal of Soil Research* **46**(1): 76-82.

Di, H. J., K. C. Cameron, J. Milne, J. J. Drewry, N. P. Smith, T. Hendry, S. Moore and B. Reijnen (2001). A mechanical hoof for simulating animal treading under controlled conditions. *New Zealand Journal of Agricultural Research* **44**(1): 111-116.

Di, H. J., K. C. Cameron, A. Podolyan and A. Robinson (2014). Effect of soil moisture status and a nitrification inhibitor, dicyandiamide, on ammonia oxidizer and denitrifier growth and nitrous oxide emissions in a grassland soil. *Soil Biology & Biochemistry* **73**: 59-68.

Di, H. J., K. C. Cameron, J. P. Shen, C. S. Winefield, M. O'Callaghan, S. Bowatte and J. Z. He (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience* **2**(9): 621-624.

Di, H. J., K. C. Cameron and R. R. Sherlock (2007). Comparison of the effectiveness of a nitrification inhibitor, dicyandiamide, in reducing nitrous oxide emissions in four different soils under different climatic and management conditions. *Soil Use and Management* **23**(1): 1-9.

Di, H. J., K. C. Cameron, R. R. Sherlock, J. P. Shen, J. Z. He and C. S. Winefield (2010). Nitrous oxide emissions from grazed grassland as affected by a nitrification inhibitor, dicyandiamide, and relationships with ammonia-oxidizing bacteria and archaea. *Journal of Soils and Sediments* **10**(5): 943-954.

Ding, Y., Y. X. Liu, W. X. Wu, D. Z. Shi, M. Yang and Z. K. Zhong (2010). Evaluation of Biochar Effects on Nitrogen Retention and Leaching in Multi-Layered Soil Columns. *Water Air and Soil Pollution* **213**(1-4): 47-55.

Dobbie, K. E., I. P. McTaggart and K. A. Smith (1999). Nitrous oxide emissions from intensive agricultural systems: Variations between crops and seasons, key driving variables, and mean emission factors. *Journal of Geophysical Research-Atmospheres* **104**(D21): 26891-26899.

Dobbie, K. E. and K. A. Smith (2003). Nitrous oxide emission factors for agricultural soils in Great Britain: the impact of soil water-filled pore space and other controlling variables. *Global Change Biology* **9**(2): 204-218.

Doole, G. J. (2014). Least-cost greenhouse gas mitigation on New Zealand dairy farms. *Nutrient Cycling in Agroecosystems* **98**(2): 235-251.

Drewry, J. J. (2006). Natural recovery of soil physical properties from treading damage of pastoral soils in New Zealand and Australia: A review. *Agriculture, Ecosystems & Environment* **114**(2-4): 159-169.

Drewry, J. J., K. C. Cameron and G. D. Buchan (2001). Effect of simulated dairy cow treading on soil physical properties and ryegrass pasture yield. *New Zealand Journal of Agricultural Research* **44**(2-3): 181-190.

Drewry, J. J., K. C. Cameron and G. D. Buchan (2008). Pasture yield and soil physical property responses to soil compaction from treading and grazing - a review. *Australian Journal of Soil Research* **46**(3): 237-256.

Drewry, J. J., R. P. Littlejohn and R. J. Paton (2000). A survey of soil physical properties on sheep and dairy farms in southern New Zealand. *New Zealand Journal of Agricultural Research* **43**(2): 251-258.

Drewry, J. J. and R. J. Paton (2005). Soil physical quality under cattle grazing of a winter-fed brassica crop. *Australian Journal of Soil Research* **43**(4): 525-531.

Drewry, J. J., R. J. Paton and R. M. Monaghan (2004). Soil compaction and recovery cycle on a Southland dairy farm: implications for soil monitoring. *Australian Journal of Soil Research* **42**(7): 851-856.

Eckard, R. J., C. Grainger and C. A. M. de Klein (2010). Options for the abatement of methane and nitrous oxide from ruminant production: A review. *Livestock Science* **130**(1-3): 47-56.

Enwall, K., L. Philippot and S. Hallin (2005). Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Applied and environmental microbiology* **71**(12): 8335-8343.

Fies, J. C., N. De Louvigny and A. Chanzy (2002). The role of stones in soil water retention. *European Journal of Soil Science* **53**(1): 95-104.

Firestone, M. K. (1982). Biological denitrification. *Nitrogen in agricultural soils*: 289-326.

Firestone, M. K. and E. A. Davidson (1989). *Microbiological basis of NO and N_2O production and consumption in soil*. Chichester UK, John Wiley & Sons.

Firestone, M. K., R. B. Firestone and J. M. Tiedje (1980). Nitrous oxide from soil denitrification - factors controlling biological production. *Science* **208**(4445): 749-751.

Fischer, D., M. Uksa, W. Tischler, T. Kautz, U. Kopke and M. Schloter (2013). Abundance of ammonia oxidizing microbes and denitrifiers in different soil horizons of an agricultural soil in relation to the cultivated crops. *Biology and Fertility of Soils* **49**(8): 1243-1246.

Fowler, D., K. Pilegaard, M. A. Sutton, P. Ambus, M. Raivonen, J. Duyzer, D. Simpson, H. Fagerli, S. Fuzzi, J. K. Schjoerring, C. Granier, A. Neftel, I. S. A. Isaksen, P. Laj, M. Maione, P. S. Monks, J. Burkhardt, U. Daemmgen, J. Neirynck, E. Personne, R. Wichink-Kruit, K. Butterbach-Bahl, C. Flechard, J. P. Tuovinen, M. Coyle, G. Gerosa, B. Loubet, N. Altimir, L. Gruenhage, C. Ammann, S. Cieslik, E. Paoletti, T. N. Mikkelsen, H. Ro-Poulsen, P. Cellier, J. N. Cape, L. Horvath, F. Loreto, U. Niinemets, P. I. Palmer, J. Rinne, P. Misztal, E. Nemitz, D. Nilsson, S. Pryor, M. W. Gallagher, T. Vesala, U. Skiba, N. Brueggemann, S. Zechmeister-Boltenstern, J. Williams, C. O'Dowd, M. C. Facchini, G. de Leeuw, A. Flossman, N. Chaumerliac and J. W. Erisman (2009). Atmospheric composition change: Ecosystems-Atmosphere interactions. *Atmospheric Environment* **43**(33): 5193-5267.

Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal and X. Perret (1997). Molecular basis of symbiosis between Rhizobium and legumes. *Nature* **387**(6631): 394-401.

Galloway, J. N. (1998). The global nitrogen cycle: changes and consequences. *Environmental pollution* **102**: 15-24.

Galloway, J. N., J. D. Aber, J. W. Erisman, S. P. Seitzinger, R. W. Howarth, E. B. Cowling and B. J. Cosby (2003). The nitrogen cascade. *Bioscience* **53**(4): 341-356.

Galloway, J. N., A. R. Townsend, J. W. Erisman, M. Bekunda, Z. C. Cai, J. R. Freney, L. A. Martinelli, S. P. Seitzinger and M. A. Sutton (2008). Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* **320**(5878): 889-892.

Geets, J., M. de Cooman, L. Wittebolle, K. Heylen, B. Vanparys, P. De Vos, W. Verstraete and N. Boon (2007). Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. *Applied microbiology and biotechnology* **75**(1): 211-221.

Granli, T. and O. C. Bockman (1994). Nitrous oxide from agriculture. *Norwegian Journal of Agricultural Sciences*: 128 pp.

Green, S. J., O. Prakash, T. M. Gihring, D. M. Akob, P. Jasrotia, P. M. Jardine, D. B. Watson, S. D. Brown, A. V. Palumbo and J. E. Kostka (2010). Denitrifying bacteria isolated from terrestrial subsurface sediments exposed to mixed-waste contamination. *Applied and environmental microbiology* **76**(10): 3244-3254.

Groffman, P. M., M. A. Altabet, J. K. Bohlke, K. Butterbach-Bahl, M. B. David, M. K. Firestone, A. E. Giblin, T. M. Kana, L. P. Nielsen and M. A. Voytek (2006). Methods for measuring denitrification: Diverse approaches to a difficult problem. *Ecological Applications* **16**(6): 2091-2122.

Grover, B. L. (1955). Simplified air permeameters for soil in place. *Soil Science Society of America. Proceedings* **19**: 414-418.

Hale, S. E., V. Alling, V. Martinsen, J. Mulder, G. D. Breedveld and G. Cornelissen (2013). The sorption and desorption of phosphate-P, ammonium-N and nitrate-N in cacao shell and corn cob biochars. *Chemosphere* **91**(11): 1612-1619.

Hallin, S. and P. E. Lindgren (1999). PCR detection of genes encoding nitrile reductase in denitrifying bacteria. *Applied and environmental microbiology* **65**(4): 1652-1657.

Hamamoto, S., P. Moldrup, K. Kawamoto and T. Komatsu (2009a). Effect of Particle Size and Soil Compaction on Gas Transport Parameters in Variably Saturated, Sandy Soils. *Vadose Zone Journal* **8**(4): 986-995.

Hamamoto, S., P. Moldrup, K. Kawamoto, T. Komatsu and D. E. Rolston (2009b). Unified Measurement System for the Gas Dispersion Coefficient, Air Permeability, and Gas Diffusion Coefficient in Variably Saturated Soil. *Soil Science Society of America Journal* **73**(6): 1921-1930.

Hamonts, K., N. Balaine, E. Moltchanova, M. Beare, S. Thomas, S. A. Wakelin, M. O'Callaghan, L. M. Condron and T. J. Clough (2013). Influence of soil bulk density and matric potential on microbial dynamics, inorganic N transformations, N_2O and N_2 fluxes following urea deposition. *Soil Biology & Biochemistry* **65**: 1-11.

Haynes, R. (1986). *Mineral nitrogen in the plant-soil system*, Elsevier.

Haynes, R. J. and P. H. Williams (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in Agronomy, Vol 49*. San Diego, Academic Press Inc. **49:** 119-199.

Henry, S., E. Baudoin, J. C. Lopez-Gutierrez, F. Martin-Laurent, A. Brauman and L. Philippot (2004). Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *Journal of microbiological methods* **59**(3): 327-335.

Henry, S., D. Bru, B. Stres, S. Hallet and L. Philippot (2006). Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Applied and environmental microbiology* **72**(8): 5181-5189.

Herbin, T., D. Hennessy, K. G. Richards, A. Piwowarczyk, J. J. Murphy and N. M. Holden (2011). The effects of dairy cow weight on selected soil physical properties indicative of compaction. *Soil Use and Management* **27**(1): 36-44.

Hewitt, A. E. (2010). New Zealand soil classification. Landcare Research Science Series. Landcare Research Lincoln NZ., Manaaki Whenua Press**:** 136 pp.

Hill, A. M. (2014). *Nitrate leaching losses and mitigation on dairy winter forage grazing systems*. Unpublished PhD theisis. Lincoln University, Lincoln, NZ.

Hojberg, O., N. P. Revsbech and J. M. Tiedje (1994). Denitrification in soil aggregates analysed with microsensors for nitrous oxide and oxygen. *Soil Science Society of America Journal* **58**(6): 1691-1698.

Hollister, C. C., J. J. Bisogni and J. Lehmann (2013). Ammonium, Nitrate, and Phosphate Sorption to and Solute Leaching from Biochars Prepared from Corn Stover (Zea mays L.) and Oak Wood (Quercus spp.). *Journal of environmental quality* **42**(1): 137-144.

Hristov, A. N., M. Hanigan, A. Cole, R. Todd, T. A. McAllister, P. M. Ndegwa and A. Rotz (2011). Review: Ammonia emissions from dairy farms and beef feedlots. *Canadian Journal of Animal Science* **91**(1): 1-35.

Hutchinson, G. L. and A. R. Mosier (1981). Improved soil cover method for field measurement of nitrous-oxide fluxes. *Soil Science Society of America Journal* **45**(2): 311-316.

IPCC (2007). Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller. Cambridge, United Kingdom, and New York, NY, USA.

IPCC (2013). Climate Change 2013: The physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P. M. Midgley. Cambridge, United Kingdom and New York**:** 1535.

Iversen, B. V., P. Moldrup and P. Loll (2004). Runoff modelling at two field slopes: use of in situ measurements of air permeability to characterize spatial variability of saturated hydraulic conductivity. *Hydrological Processes* **18**(5): 1009-1026.

Iversen, B. V., P. Moldrup, P. Schjonning and O. H. Jacobsen (2003). Field Application of a Portable Air Permeameter to Characterize Spatial Variability in Air and Water Permeability. *Vadose Zone Journal* **2**(4): 618-626.

Iversen, B. V., P. Schjonning, T. G. Poulsen and P. Moldrup (2001). In situ, on-site and laboratory measurements of soil air permeability: Boundary conditions and measurement scale. *Soil Science* **166**(2): 97-106.

Jalbert, M. and J. H. Dane (2003). A Handheld Device for Intrusive and Nonintrusive Field Measurements of Air Permeability. *Vadose Zone Journal* **2**(4): 611-617.

Jayakumar, A., G. D. O'Mullan, S. W. Naqvi and B. B. Ward (2009). Denitrifying bacterial community composition changes associated with stages of denitrification in oxygen minimum zones. *Microbial ecology* **58**(2): 350-362.

Jeffery, S., F. G. A. Verheijen, M. van der Velde and A. C. Bastos (2011). A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. *Agriculture Ecosystems & Environment* **144**(1): 175-187.

Jenkinson, B., G. Edwards and R. Bryant (2014). *Grazing behaviour, dry matter intake and urination patterns of dairy cows offered kale or fodder beet in winter*. Proceedings of the New Zealand Society of Animal Production.

Jetten, M. S. (2008). The microbial nitrogen cycle. *Environmental microbiology* **10**(11): 2903-2909.

Jia, Z. and R. Conrad (2009). Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental microbiology* **11**(7): 1658-1671.

Jones, C. M., D. R. Graf, D. Bru, L. Philippot and S. Hallin (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *The ISME journal* **7**(2): 417-426.

Jones, C. M., A. Spor, F. P. Brennan, M. C. Breuil, D. Bru, P. Lemanceau, B. Griffiths, S. Hallin and L. Philippot (2014). Recently identified microbial guild mediates soil N_2O sink capacity. *Nature Climate Change* **4**(9): 801-805.

Kandeler, E., K. Deiglmayr, D. Tscherko, D. Bru and L. Philippot (2006). Abundance of narG, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Applied and environmental microbiology* **72**(9): 5957-5962.

Karer, J., B. Wimmer, F. Zehetner, S. Kloss and G. Soja (2013). Biochar application to temperate soils: effects on nutrient uptake and crop yield under field conditions. *Agricultural and Food Science* **22**(4): 390-403.

Kelliher, F. M., T. J. Clough, H. Clark, G. Rys and J. R. Sedcole (2008). The temperature dependence of dicyandiamide (DCD) degradation in soils: A data synthesis. *Soil Biology & Biochemistry* **40**(7): 1878-1882.

Kelliher, F. M., N. Cox, T. J. van der Weerden, C. A. M. de Klein, J. Luo, K. C. Cameron, H. J. Di, D. Giltrap and G. Rys (2014). Statistical analysis of nitrous oxide emission factors from pastoral agriculture field trials conducted in New Zealand. *Environmental pollution* **186**: 63-66.

Kelliher, F. M., A. R. Reisinger, R. J. Martin, M. J. Harvey, S. J. Price and R. R. Sherlock (2002). Measuring nitrous oxide emission rate from grazed pasture using Fourier-transform infrared spectroscopy in the nocturnal boundary layer. *Agricultural and Forest Meteorology* **111**(1): 29-38.

Khelil-Arfa, H., A. Boudon, G. Maxin and P. Faverdin (2012). Prediction of water intake and excretion flows in Holstein dairy cows under thermoneutral conditions. *Animal* **6**(10): 1662-1676.

Kirkham, D. (1947). Field method for determination of air permeability of soil in its undisturbed state. *Proceedings. Soil Science Society of America* **11**: 93-99.

Kleinman, P. J. A., and K. Soder (2008). The impact of hybrid dairy systems on air, soil and water quality: focus on nitrogen and phosphorus cycling. *Environmental Impacts of Pasture-based Farming*. R.W. Mc Dowell (eds). Wallingford UK, CAB International. 249-276.

Kloos, K., A. Mergel, C. Rosch and H. Bothe (2001). Denitrification within the genus Azospirillum and other associative bacteria. *Australian Journal of Plant Physiology* **28**(9): 991-998.

Knowles, R. (1982). Denitrification. *Microbiological Reviews* **46**(1): 43-70.

Kool, D. M., J. Dolfing, N. Wrage and J. W. Van Groenigen (2011). Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. *Soil Biology & Biochemistry* **43**(1): 174-178.

Koops, J. G., M. L. vanBeusichem and O. Oenema (1997). Nitrogen loss from grassland on peat soils through nitrous oxide production. *Plant and Soil* **188**(1): 119-130.

Kraft, B., M. Strous and H. E. Tegetmeyer (2011). Microbial nitrate respiration--genes, enzymes and environmental distribution. *Journal of biotechnology* **155**(1): 104-117.

Lantinga, E. A., J. A. Keuning, J. Groenwold and P. J. Deenen (1987). Distribution of excreted nitrogen by grazing cattle and its effects on sward quality, herbage production and utilization. *Animal Manure on Grassland and Fodder Crops. Fertilizer or Waste?* Springer, Netherlands, 103.117.

Laughlin, R. J., T. Rutting, C. Mueller, C. J. Watson and R. J. Stevens (2009). Effect of acetate on soil respiration, N_2O emissions and gross N transformations related to fungi and bacteria in a grassland soil. *Applied Soil Ecology* **42**(1): 25-30.

Laughlin, R. J. and R. J. Stevens (2003). Changes in composition of ^{15}N-labeled gases during storage in septum-capped vials. *Soil Science Society of America Journal* **67**(2): 540-543.

Le Roux, X., B. Schmid, F. Poly, R. L. Barnard, P. A. Niklaus, N. Guillaumaud, M. Habekost, Y. Oelmann, L. Philippot, J. F. Salles, M. Schloter, S. Steinbeiss and A. Weigelt (2013). Soil Environmental

Conditions and Microbial Build-Up Mediate the Effect of Plant Diversity on Soil Nitrifying and Denitrifying Enzyme Activities in Temperate Grasslands. *Plos One* **8**(4): 11.

Le Treut, H., R. Somerville, U. Cubasch, Y. Ding, C. Mauritzen, A. Mokssit, T. Peterson and M. Prather (2007). Chapter 1: Historical Overview of Climate Change. *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor, and H. L. Miller (eds). Cambridge University Press.

Ledgard, S., R. Schils, J. Ericksen and J. Lou (2009). Environmental impacts of grazed clover/grass pastures. *Irish Journal of Agricultural and Food Research* **48**(2): 209-226.

Lee, J. W., M. Kidder, B. R. Evans, S. Paik, A. C. Buchanan, C. T. Garten and R. C. Brown (2010). Characterization of Biochars Produced from Cornstovers for Soil Amendment. *Environmental Science & Technology* **44**(20): 7970-7974.

Lehmann, J. (2007a). Bio-energy in the black. *Frontiers in Ecology and the Environment* **5**(7): 381-387.

Lehmann, J. (2007b). A handful of carbon. *Nature* **447**(7141): 143-144.

Lehmann, J. and S. Joseph (2009). Biochar for environmental management: An introduction. *Biochar for environmental management, science and technology.* J. Lehmann and S. Joseph (eds). London, Earthscan**:** 1-12.

Lehmann, J., M. C. Rillig, J. Thies, C. A. Masiello, W. C. Hockaday and D. Crowley (2011). Biochar effects on soil biota – A review. *Soil Biology and Biochemistry*.

Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster and C. Schleper (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442**(7104): 806-809.

Levy-Booth, D. J. and R. S. Winder (2010). Quantification of nitrogen reductase and nitrite reductase genes in soil of thinned and clear-cut Douglas-fir stands by using real-time PCR. *Applied and environmental microbiology* **76**(21): 7116-7125.

Li, S., H. Deng, C. Rensing and Y. G. Zhu (2014). Compaction stimulates denitrification in an urban park soil using ^{15}N tracing technique. *Environmental Science and Pollution Research* **21**(5): 3783-3791.

Liang, P., C. G. Bowers and H. D. Bowen (1995). Finite element model to determine the shape factor for soil air permeability measurements. *Transactions of the Asae* **38**(4): 997-1003.

Lilburne, L., S. Carrick, T. Webb and J. Moir (2012). Computer-based evaluation of methods to sample nitrate leached from grazed pasture. *Soil Use and Management* **28**(1): 19-26.

Lilly, A., B. C. Ball, I. P. McTaggart and P. L. Horne (2003). Spatial and temporal scaling of nitrous oxide emissions from the field to the regional scale in Scotland. *Nutrient Cycling in Agroecosystems* **66**(3): 241-257.

Linn, D. M. and J. W. Doran (1984). Effect of Water-Filled Pore Space on Carbon Dioxide and Nitrous Oxide Production in Tilled and Nontilled Soils. *Soil Science Society of America Journal* **48**: 1267-1272.

Lipiec, J. and R. Hatano (2003). Quantification of compaction effects on soil physical properties and crop growth. *Geoderma* **116**(1-2): 107-136.

Loll, P., P. Moldrup, P. Schjonning and H. Riley (1999). Predicting saturated hydraulic conductivity from air permeability: Application in stochastic water infiltration modeling. *Water Resources Research* **35**(8): 2387-2400.

Lopez-Gutierrez, J. C., S. Henry, S. Hallet, F. Martin-Laurent, G. Catroux and L. Philippot (2004). Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *Journal of microbiological methods* **57**(3): 399-407.

Lovell, R. D. and S. C. Jarvis (1996). Effects of urine on soil microbial biomass, methanogenesis, nitrification and denitrification in grassland soils. *Plant and Soil* **186**(2): 265-273.

Luo, J., C. A. M. de Klein, S. F. Ledgard and S. Saggar (2010). Management options to reduce nitrous oxide emissions from intensively grazed pastures: A review. *Agriculture, Ecosystems & Environment* **136**(3-4): 282-291.

Luo, J., S. F. Ledgard, C. A. M. de Klein, S. B. Lindsey and M. Kear (2008a). Effects of dairy farming intensification on nitrous oxide emissions. *Plant and Soil* **309**(1-2): 227-237.

Luo, J., S. F. Ledgard and S. B. Lindsey (2007). Nitrous oxide emissions from application of urea on New Zealand pasture. *New Zealand Journal of Agricultural Research* **50**(1): 1-11.

Luo, J., S. F. Ledgard and S. B. Lindsey (2008b). A test of a winter farm management option for mitigating nitrous oxide emissions from a dairy farm. *Soil Use and Management* **24**(2): 121-130.

Luo, J., S. B. Lindsey and S. F. Ledgard (2008c). Nitrous oxide emissions from animal urine application on a New Zealand pasture. *Biology and Fertility of Soils* **44**(3): 463-470.

Luo, J. and S. Saggar (2008). Nitrous oxide and methane emissions from a dairy farm stand-off pad. *Australian Journal of Experimental Agriculture* **48**(1-2): 179-182.

Luo, J., S. Saggar, R. Bhandral, N. Bolan, S. Ledgard, S. Lindsey and W. Sun (2008d). Effects of irrigating dairy-grazed grassland with farm dairy effluent on nitrous oxide emissions. *Plant and Soil* **309**(1-2): 119-130.

Luo, J., R. W. Tillman and P. R. Ball (2000). Nitrogen loss through denitrification in a soil under pasture in New Zealand. *Soil Biology and Biochemistry* **32**(4): 497-509.

Luo, J., R. W. Tillman, R. E. White and P. R. Ball (1998). Variation in denitrification activity with soil depth under pasture. *Soil Biology & Biochemistry* **30**(7): 897-903.

Luo, J., R. E. White, P. R. Ball and R. W. Tillman (1996). Measuring denitrification activity in soils under pasture: Optimizing conditions for the short-term denitrification enzyme assay and effects of soil storage on denitrification activity. *Soil Biology & Biochemistry* **28**(3): 409-417.

Luo, J. F., C. Hoogendoorn, T. van der Weerden, S. Saggar, C. de Klein, D. Giltrap, M. Rollo and G. Rys (2013a). Nitrous oxide emissions from grazed hill land in New Zealand. *Agriculture Ecosystems & Environment* **181**: 58-68.

Luo, J. F., S. F. Ledgard and S. B. Lindsey (2013b). Nitrous oxide and greenhouse gas emissions from grazed pastures as affected by use of nitrification inhibitor and restricted grazing regime. *Science of the Total Environment* **465**: 107-114.

Ma, D. and M. Shao (2008). Simulating infiltration into stony soils with a dual-porosity model. *European Journal of Soil Science* **59**(5): 950-959.

Maasland, M. and D. Kirkham (1955). Theory and measurement of anisotropic air permeability in soil. *Soil Science Society of America. Proceedings* **19**: 395-400.

Maeda, K., R. Morioka, D. Hanajima and T. Osada (2010). The Impact of Using Mature Compost on Nitrous Oxide Emission and the Denitrifier Community in the Cattle Manure Composting Process. *Microbial ecology* **59**(1): 25-36.

Maier, M. and H. Schack-Kirchner (2014). Using the gradient method to determine soil gas flux: A review. *Agricultural and Forest Meteorology* **192**: 78-95.

Majumdar, D. (2002). Suppression of nitrification and N_2O emission by karanjin - a nitrification inhibitor prepared from karanja (Pongamia glabra Vent.). *Chemosphere* **47**(8): 845-850.

Malcolm, B. J. (2013). *The effect of pasture species composition and a nitrification inhibitor on nitrate leaching losses*. Unpublished PhD thesis. Lincoln University, Lincoln, NZ.

Mathieu, O., J. Lévêque, C. Hénault, M. J. Milloux, F. Bizouard and F. Andreux (2006). Emissions and spatial variability of N_2O, N_2 and nitrous oxide mole fraction at the field scale, revealed with ^{15}N isotopic techniques. *Soil Biology and Biochemistry* **38**(5): 941-951.

Mazurak, A. P. and R. E. Ramig (1962). Aggregation and air-water permeabilities in a chernozem soil cropped to perennial grasses and fallow-grain. *Soil Science* **94**: 151-157.

McDowell, R., J. Drewry, R. Muirhead and R. Paton (2003). Cattle treading and phosphorus and sediment loss in overland flow from grazed cropland. *Soil Research* **41**(8): 1521-1532.

Menneer, J., S. Ledgard, C. McLay and W. Silvester (2005). Animal treading stimulates denitrification in soil under pasture. *Soil Biology and Biochemistry* **37**(9): 1625-1629.

Michotey, V., V. Mejean and P. Bonin (2000). Comparison of methods for quantification of cytochrome cd(1)-denitrifying bacteria in environmental marine samples. *Applied and environmental microbiology* **66**(4): 1564-1571.

Ministry for the Environment (2011). New Zealand's Greenhouse Gas Inventory 1990-2009. Ministry for the Environment. Wellington, New Zealand, Ministry for the Environment**:** 387.

Ministry for the Environment (2013). New Zealand's Greenhouse Gas Inventory 1990-2011. Ministry. for. the Environment. Wellington, New Zealand, Ministry for the Environment**:** 424.

Ministry for the Environment (2014). New Zealand's Greenhouse Gas Inventory 1990-2012. Ministry. for. the Environment. Wellington, New Zealand, Ministry for the Environment**:** 412.

Moir, J. L., K. C. Cameron, H. J. Di and U. Fertsak (2011). The spatial coverage of dairy cattle urine patches in an intensively grazed pasture system. *Journal of Agricultural Science* **149**: 473-485.

Moir, J. L., B. J. Malcolm, K. C. Cameron and H. J. Di (2012). The effect of dicyandiamide on pasture nitrate concentration, yield and N offtake under high N loading in winter and spring. *Grass and Forage Science* **67**(3): 391-402.

Moldrup, P., T. Olesen, T. Komatsu, P. Schjonning and D. E. Rolston (2001). Tortuosity, diffusivity, and permeability in the soil liquid and gaseous phases. *Soil Science Society of America Journal* **65**(3): 613-623.

Moldrup, P., T. G. Poulsen, P. Schjonning, T. Olesen and T. Yamaguchi (1998). Gas permeability in undisturbed soils: Measurements and predictive models. *Soil Science* **163**(3): 180-189.

Monaghan, R. M. and D. Barraclough (1993). Nitrous oxide and dinitrogen emission from urine-affected soil under controlled conditions. *Plant and Soil* **151**(1): 127-138.

Monaghan, R. M., L. C. Smith and C. A. M. de Klein (2013). The effectiveness of the nitrification inhibitor dicyandiamide (DCD) in reducing nitrate leaching and nitrous oxide emissions from a grazed winter forage crop in southern New Zealand. *Agriculture Ecosystems & Environment* **175**: 29-38.

Monaghan, R. M., R. J. Wilcock, L. C. Smith, B. Tikkisetty, B. S. Thorrold and D. Costall (2007). Linkages between land management activities and water quality in an intensively farmed catchment in southern New Zealand. *Agriculture Ecosystems & Environment* **118**(1-4): 211-222.

Monteny, G.-J., A. Bannink and D. Chadwick (2006). Greenhouse gas abatement strategies for animal husbandry. *Agriculture, Ecosystems & Environment* **112**(2-3): 163-170.

Mooney, S. J. and W. Nipattasuk (2003). Quantification of the effects of soil compaction on water flow using dye tracers and image analysis. *Soil Use and Management* **19**(4): 356-363.

Mosier, A., C. Kroeze, C. Nevison, O. Oenema, S. Seitzinger and O. van Cleemput (1998). Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle. *Nutrient Cycling in Agroecosystems* **52**(2): 225-248.

Mounier, E., S. Hallet, D. Cheneby, E. Benizri, Y. Gruet, C. Nguyen, S. Piutti, C. Robin, S. Slezack-Deschaumes, F. Martin-Laurent, J. C. Germon and L. Philippot (2004). Influence of maize mucilage on the diversity and activity of the denitrifying community. *Environmental microbiology* **6**(3): 301-312.

Muller, T. N. and M. E. Hamilton (1992). A simple, effective method for determining the bulk density of stony soils. *Communications in Soil Science and Plant Analysis* **23**(3-4): 313-319.

Myrold, D. D. (1998). Transformations of nitrogen. *Principles and applications of soil microbiology*. D. M. Sylvia, J. J. Furhrmann, P. G. Hartel and D. A. Zuberer (eds).Prentice Hall, New Jersey, USA.

Nearing, M. A., J. R. Simanton, L. D. Norton, S. J. Bulygin and J. Stone (1999). Soil erosion by surface water flow on a stony, semiarid hillslope. *Earth Surface Processes and Landforms* **24**(8): 677-686.

Nie, Z. N., G. N. Ward and A. T. Michael (2001). Impact of pugging by dairy cows on pastures and indicators of pugging damage to pasture soil in south-western Victoria. *Australian Journal of Agricultural Research* **52**(1): 37-43.

Nittami, T., T. Magura, Y. Imai and K. Matsumoto (2009). Influence of the electron acceptor on nitrite reductase gene (nir) diversity in an activated sludge community. *Journal of bioscience and bioengineering* **108**(5): 394-399.

Oakley, B. B., C. A. Francis, K. J. Roberts, C. A. Fuchsman, S. Srinivasan and J. T. Staley (2007). Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. *Environmental microbiology* **9**(1): 118-130.

Oenema, O., G. L. Velthof, S. Yamulki and S. C. Jarvis (1997). Nitrous oxide emission from grazed grassland. *Soil Use and Management* **13**(4): 288-295.

Oudshoorn, F. W., T. Kristensen and E. S. Nadimi (2008). Dairy cow defecation and urination frequency and spatial distribution in relation to time-limited grazing. *Livestock Science* **113**(1): 62-73.

Pande, T. N. and H. Yamamoto (2006). Cattle treading effects on plant growth and soil stability in the mountain grassland of Japan. *Land Degradation & Development* **17**(4): 419-428.

Paranychianakis, N. V., M. Tsiknia, G. Giannakis, N. P. Nikolaidis and N. Kalogerakis (2013). Nitrogen cycling and relationships between ammonia oxidizers and denitrifiers in a clay-loam soil. *Applied microbiology and biotechnology* **97**(12): 5507-5515.

Peng, X., L. L. Ye, C. H. Wang, H. Zhou and B. Sun (2011). Temperature- and duration-dependent rice straw-derived biochar: Characteristics and its effects on soil properties of an Ultisol in southern China. *Soil & Tillage Research* **112**(2): 159-166.

Philippot, L. (2002). Denitrifying genes in bacterial and Archaeal genomes. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1577**(3): 355-376.

Philippot, L., J. Andert, C. M. Jones, D. Bru and S. Hallin (2011). Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. *Global Change Biology* **17**(3): 1497-1504.

Philpott, H. G. (1937). *A History of the New Zealand Dairy Industry*. Wellington, N.Z., G.H. Loney, Government Printer.

Pleasants, A. B., P. R. Shorten and G. C. Wake (2007). The distribution of urine deposited on a pasture from grazing animals. *Journal of Agricultural Science* **145**: 81-86.

Poulsen, T. G., B. V. Iversen, T. Yamaguchi, P. Moldrup and P. Schjonning (2001). Spatial and temporal dynamics of air permeability in a constructed field. *Soil Science* **166**(3): 153-162.

Prather, M. J. and J. Hsu (2010). Coupling of Nitrous Oxide and Methane by Global Atmospheric Chemistry. *Science* **330**(6006): 952-954.

Randrup, T. B. and J. M. Lichter (2001). Measuring soil compaction on construction sites: a review of surface nuclear gauges and penetrometers. *Journal of Arboriculture* **27**(3): 109-117.

Reinhart, K. G. (1961). The problem of stones in soil-moisture measurement. *Soil Science Society of America. Proceedings* **25**: 268-270.

Renault, P. and P. Stengel (1994). Modeling oxygen diffusion in aggregated soils. 1. Anaerobiosis inside the aggregates. *Soil Science Society of America Journal* **58**(4): 1017-1023.

Reyna, L., D. A. Wunderlin and S. Genti-Raimondi (2010). Identification and quantification of a novel nitrate-reducing community in sediments of Suquia River basin along a nitrate gradient. *Environmental pollution* **158**(5): 1608-1614.

Robertson, G. P. and J. M. Tiedje (1987). Nitrous oxide sources in aerobic soils - nitrification, denitrification and other biological processes. *Soil Biology & Biochemistry* **19**(2): 187-193.

Robertson, G. P. and P. M. Vitousek (2009). Nitrogen in Agriculture: Balancing the Cost of an Essential Resource. *Annual Review of Environment and Resources* **34**(1): 97-125.

Robinson, A., H. J. Di, K. C. Cameron and A. Podolyan (2014). Effect of soil aggregate size and dicyandiamide on N_2O emissions and ammonia oxidizer abundance in a grazed pasture soil. *Soil Use and Management* **30**(2): 231-240.

Rotthauwe, J. H., K. P. Witzel and W. Liesack (1997). The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and environmental microbiology* **63**(12): 4704-4712.

Ruser, R., H. Flessa, R. Russow, G. Schmidt, F. Buegger and J. C. Munch (2006). Emission of N_2O, N_2 and CO_2 from soil fertilized with nitrate: Effect of compaction, soil moisture and rewetting. *Soil Biology & Biochemistry* **38**(2): 263-274.

Russo, D. (1983). Leaching characteristics of a stony desert soil. *Soil Science Society of America Journal* **47**(3): 431-438.

Saarijarvi, K. and P. Virkajarvi (2009). Nitrogen dynamics of cattle dung and urine patches on intensively managed boreal pasture. *Journal of Agricultural Science* **147**: 479-491.

Saggar, S., N. Jha, J. Deslippe, N. S. Bolan, J. Luo, D. L. Giltrap, D. G. Kim, M. Zaman and R. W. Tillman (2013). Denitrification and N_2O:N_2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. *Science of the Total Environment* **465**: 173-195.

Saggar, S., J. Luo, D. Giltrap and M. Maddena (2009). Nitrous oxide emissions from temperate grasslands: processes, measurements, modelling and mitigation. *Nitrous oxide emissions research progress. Environmental Science, Engineering and Technology Series. Nova Science Publishers, New York, USA.[Links]*: 1-66.

Sanchez-Giron, V., E. Andreu and J. L. Hernanz (1998). Response of five types of soil to simulated compaction in the form of confined uniaxial compression tests. *Soil & Tillage Research* **48**(1-2): 37-50.

Sanford, R. A., D. D. Wagner, Q. Z. Wu, J. C. Chee-Sanford, S. H. Thomas, C. Cruz-Garcia, G. Rodriguez, A. Massol-Deya, K. K. Krishnani, K. M. Ritalahti, S. Nissen, K. T. Konstantinidis and F. E. Loffler (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proceedings of the National Academy of Sciences of the United States of America* **109**(48): 19709-19714.

Sarkhot, D. V., T. A. Ghezzehei and A. A. Berhe (2013). Effectiveness of Biochar for Sorption of Ammonium and Phosphate from Dairy Effluent. *Journal of environmental quality* **42**(5): 1545-1554.

Scheer, C., P. R. Grace, D. W. Rowlings, S. Kimber and L. Van Zwieten (2011). Effect of biochar amendment on the soil-atmospere exchange of greenhouse gases from an intensive subtropical pasture in northern New South Wales, Australia. *Plant and Soil* **345**: 47-58.

Schjonning, P., I. K. Thomsen, P. Moldrup and B. T. Christensen (2003). Linking soil microbial activity to water- and air-phase contents and diffusivities. *Soil Science Society of America Journal* **67**(1): 156-165.

Schmidt, M. W. I., M. S. Torn, S. Abiven, T. Dittmar, G. Guggenberger, I. A. Janssens, M. Kleber, I. Kogel-Knabner, J. Lehmann, D. A. C. Manning, P. Nannipieri, D. P. Rasse, S. Weiner and S. E. Trumbore (2011). Persistence of soil organic matter as an ecosystem property. *Nature* **478**(7367): 49-56.

Schulin, R., P. J. Wierenga, H. Fluhler and J. Leuenberger (1987). Solute transport through a stony soil. *Soil Science Society of America Journal* **51**(1): 36-42.

Selbie, D., K. Cameron, H. Di, J. Moir, G. Lanigan and K. Richards (2014). The effect of urinary nitrogen loading rate and a nitrification inhibitor on nitrous oxide emissions from a temperate grassland soil. *Journal of Agricultural Science*.

Sexstone, A. J., N. P. Revsbech, T. B. Parkin and J. M. Tiedje (1985). Direct measurement of oxygen profiles and denitrification rates in soil aggregates. *Soil Science Society of America Journal* **49**(3): 645-651.

Simek, M., P. Brucek, J. Hynst, E. Uhlirova and S. Petersen (2006). Effects of excretal returns and soil compaction on nitrous oxide emissions from a cattle overwintering area. *Agriculture, Ecosystems & Environment* **112**(2-3): 186-191.

Simek, M. and J. E. Cooper (2002). The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. *European Journal of Soil Science* **53**(3): 345-354.

Singh, B. P., B. J. Hatton, B. Singh, A. L. Cowie and A. Kathuria (2010). Influence of Biochars on Nitrous Oxide Emission and Nitrogen Leaching from Two Contrasting Soils. *Journal of environmental quality* **39**(4): 1224-1235.

Singh, J., S. Saggar and N. S. Bolan (2009). Influence of dicyandiamide on nitrogen transformation and losses in cow-urine-amended soil cores from grazed pasture. *Animal Production Science* **49**(3): 253-261.

Singleton, P. L., M. Boyes and B. Addison (2000). Effect of treading by dairy cattle on topsoil physical conditions for six contrasting soil types in Waikato and Northland, New Zealand, with implications for monitoring. *New Zealand Journal of Agricultural Research* **43**(4): 559-567.

Smith, C. J., D. B. Nedwell, L. F. Dong and A. M. Osborn (2007). Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. *Applied and environmental microbiology* **73**(11): 3612-3622.

Smith, L. C., C. A. M. de Klein and W. D. Catto (2008a). Effect of dicyandiamide applied in a granular form on nitrous oxide emissions from a grazed dairy pasture in Southland, New Zealand. *New Zealand Journal of Agricultural Research* **51**(4): 387-396.

Smith, L. C., C. A. M. de Klein, R. M. Monaghan and W. D. Catto (2008b). The effectiveness of dicyandiamide in reducing nitrous oxide emissions from a cattle-grazed, winter forage crop in Southland, New Zealand. *Australian Journal of Experimental Agriculture* **48**(1-2): 160-164.

Sohi, S. P., E. Krull, E. Lopez-Capel and R. Bol (2010). A review of biochar and its use and function in soil. *Advances in Agronomy, Vol 105*. D. L. Sparks. **105:** 47-82.

Soil Survey Staff (2010). Keys to Soil Taxonomy. USDA-Natural Resources Conservation Service. Washinton, DC.

Spek, J. W., A. Bannink, G. Gort, W. H. Hendriks and J. Dijkstra (2012). Effect of sodium chloride intake on urine volume, urinary urea excretion, and milk urea concentration in lactating dairy cattle. *Journal of Dairy Science* **95**(12): 7288-7298.

Spittlehouse, D. L. (2000). Using time domain reflectometry in stony forest soil. *Canadian Journal of Soil Science* **80**(1): 3-11.

Statistics New Zealand (2014). Global New Zealand - International trade, investment, and travel profile: Year ended June 2014. Wellington, NZ., Ministry of Foreign Affairs and Trade, and Statistics New Zealand.

Steinbeiss, S., G. Gleixner and M. Antonietti (2009). Effect of biochar amendment on soil carbon balance and soil microbial activity. *Soil Biology & Biochemistry* **41**(6): 1301-1310.

Steinfeld, H., P. Gerber, T. Wassenaar, V. Castel, M. Rosales and C. d. Haan (2006). *Livestock's long shadow: environmental issues and options*. Rome Italy, Food and Agriculture Organization of the United Nations (FAO).

Suddick, E. C. and J. Six (2013). An estimation of annual nitrous oxide emissions and soil quality following the amendment of high temperature walnut shell biochar and compost to a small scale vegetable crop rotation. *The Science of the total environment* **465**: 298-307.

Syakila, A. and C. Kroeze (2011). The global nitrous oxide budget revisited. *Greenhouse Gas Measurement and Management* **1**(1): 17-26.

Taghizadeh-Toosi, A., T. J. CLough, L. M. Condron, R. R. Sherlock, C. R. Anderson and R. A. Craigie (2011). Biochar Incorporation into Pasture Soil Suppresses in situ Nitrous Oxide Emissions from Ruminant Urine Patches. *Journal of environmental quality* **40**(2): 468-476.

Taghizadeh-Toosi, A., T. J. Clough, R. R. Sherlock and L. M. Condron (2012). Biochar adsorbed ammonia is bioavailable. *Plant and Soil* **350**(1-2): 57-69.

Tatti, E., C. Goyer, B. J. Zebarth, D. L. Burton, L. Giovannetti and C. Viti (2013). Short-Term Effects of Mineral and Organic Fertilizer on Denitrifiers, Nitrous Oxide Emissions and Denitrification in Long-Term Amended Vineyard Soils. *Soil Science Society of America Journal* **77**(1): 113-122.

Theerachat, M., C. Virunanon, S. Chulalaksananukul, N. Sinbuathong and W. Chulalaksananukul (2011). *nirK* and *nirS* Nitrite reductase genes from non-agricultural forest soil bacteria in Thailand. *World journal of microbiology & biotechnology* **27**(4): 999-1003.

Thomas, S. M., M. H. Beare, G. S. Francis, H. E. Barlow and D. I. Hedderley (2008). Effects of tillage, simulated cattle grazing and soil moisture on N(2)O emissions from a winter forage crop. *Plant and Soil* **309**(1-2): 131-145.

Throback, I. N., K. Enwall, A. Jarvis and S. Hallin (2004). Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS microbiology ecology* **49**(3): 401-417.

Tyner, J. S., W. C. Wright, J. Lee and A. D. Crenshaw (2005). A dynamic air permeameter for coarse-textured soil columns and cores. *Vadose Zone Journal* **4**(2): 428-433.

van der Weerden, T. J., F. M. Kelliher and C. A. M. de Klein (2012a). Influence of pore size distribution and soil water content on nitrous oxide emissions. *Soil Research* **50**(2): 125-135.

van der Weerden, T. J., T. M. Styles and Nzga (2012b). Reducing nitrous oxide emissions from grazed winter forage crops. *Proceedings of the New Zealand Grassland Association, Vol 74*. Mosgiel, New Zealand Grassland Assoc. **74:** 57-62.

van Groenigen, J. W., P. J. Kuikman, W. J. M. de Groot and G. L. Velthof (2005a). Nitrous oxide emission from urine-treated soil as influenced by urine composition and soil physical conditions. *Soil Biology & Biochemistry* **37**(3): 463-473.

van Groenigen, J. W., G. L. Velthof, F. J. E. v. d. Bolt, A. Vos and P. J. Kuikman (2005b). Seasonal variation in N_2O emissions from urine patches: Effects of urine concentration, soil compaction and dung. *Plant and Soil* **273**(1-2): 15-27.

Van Zwieten, L., S. Kimber, S. Morris, K. Y. Chan, A. Downie, J. Rust, S. Joseph and A. Cowie (2010a). Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. *Plant and Soil* **327**(1-2): 235-246.

Van Zwieten, L., S. Kimber, S. Morris, A. Downie, E. Berger, J. Rust and C. Scheer (2010b). Influence of biochars on flux of N_2O and CO_2 from Ferrosol. *Australian Journal of Soil Research* **48**: 555-568.

Vincent, K. R. and O. A. Chadwick (1994). Synthesising bulk density for soils with abundant rock fragments. *Soil Science Society of America Journal* **58**(2): 455-464.

Wachendorf, C., C. Lampe, F. Taube and K. Dittert (2008). Nitrous oxide emissions and dynamics of soil nitrogen under ^{15}N-labeled cow urine and dung patches on a sandy grassland soil. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **171**(2): 171-180.

Wairiu, M., C. E. Mullins and C. D. Campbell (1993). Soil physical factors affecting the growth of sycamore (acer-pseudoplanatus L) in a silvopastoral system on a stony upland soil in northeast Scotland. *Agroforestry Systems* **24**(3): 295-306.

Wallenstein, M. D., D. D. Myrold, M. Firestone and M. Voytek (2006). Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications* **16**(6): 2143-2152.

Wallenstein, M. D. and R. J. Vilgalys (2005). Quantitative analyses of nitrogen cycling genes in soils. *Pedobiologia* **49**(6): 665-672.

Wang, J. Y., M. Zhang, Z. Q. Xiong, P. L. Liu and G. X. Pan (2011). Effects of biochar addition on N_2O and CO_2 emissions from two paddy soils. *Biology and Fertility of Soils* **47**(8): 887-896.

Webb, T. and C. M. Bennett (1986). Soils of Ashley Dene. Soil Bureau, DSIR, Lincoln, NZ.

Weier, K. L., J. W. Doran, J. F. Power and D. T. Walters (1993). Denitrification and the dinitrogen nitrous oxide ratio as affected by soil water, available carbon, and nitrate. *Soil Science Society of America Journal* **57**(1): 66-72.

Weiske, A., G. Benckiser, T. Herbert and J. Ottow (2001). Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. *Biology and Fertility of Soils* **34**(2): 109-117.

Wells, T., S. Fityus, D. W. Smith and H. Moe (2006). The indirect estimation of saturated hydraulic conductivity of soils, using measurements of gas permeability. I. Laboratory testing with dry granular soils. *Australian Journal of Soil Research* **44**(7): 719-725.

Whitehead, D. C. (1995). *Grassland nitrogen*, CAB international.

Williams, P. H. and R. J. Haynes (1994). Comparison of initial wetting pattern, nutrient concentraions in soil solution and the fate of ^{15}N labelled urine in sheep and cattle urine patch areas of pasture soil. *Plant and Soil* **162**(1): 49-59.

Woolf, D., J. E. Amonette, F. A. Street-Perrott, J. Lehmann and S. Joseph (2010). Sustainable biochar to mitigate global climate change. *Nature Communications* **1**: 9.

Wrage, N., G. L. Velthof, H. J. Laanbroek and O. Oenema (2004). Nitrous oxide production in grassland soils: assessing the contribution of nitrifier denitrification. *Soil Biology & Biochemistry* **36**(2): 229-236.

Wrage, N., G. L. Velthof, M. L. van Beusichem and O. Oenema (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry* **33**(12-13): 1723-1732.

Wright, J. (2013). *Water Quality in New Zealand: Land use and nutrient pollution*. The Parliamentary Commissioner for the Environment, Wellington, NZ. 82.

Wu, L., D. L. Osmond, A. K. Graves, M. R. Burchell and O. W. Duckworth (2012). Relationships Between Nitrogen Transformation Rates and Gene Abundance in a Riparian Buffer Soil. *Environmental Management* **50**(5): 861-874.

Xue, K., L. Y. Wu, Y. Deng, Z. L. He, J. Van Nostrand, P. G. Robertson, T. M. Schmidt and J. Z. Zhou (2013). Functional Gene Differences in Soil Microbial Communities from Conventional, Low-Input, and Organic Farmlands. *Applied and environmental microbiology* **79**(4): 1284-1292.

Yan, T. F., M. W. Fields, L. Y. Wu, Y. G. Zu, J. M. Tiedje and J. Z. Zhou (2003). Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. *Environmental microbiology* **5**(1): 13-24.

Yang, W. H., A. C. McDowell, P. D. Brooks and W. L. Silver (2014). New high precision approach for measuring ^{15}N-N_2 gas fluxes from terrestrial ecosystems. *Soil Biology & Biochemistry* **69**: 234-241.

Yoshida, M., S. Ishii, S. Otsuka and K. Senoo (2009). Temporal shifts in diversity and quantity of *nirS* and *nirK* in a rice paddy field soil. *Soil Biology and Biochemistry* **41**(10): 2044-2051.

Yoshida, M., S. Ishii, S. Otsuka and K. Senoo (2010). *nirK*-Harboring Denitrifiers Are More Responsive to Denitrification- Inducing Conditions in Rice Paddy Soil Than *nirS*-Harboring Bacteria. *Microbes and Environments* **25**(1): 45-48.

Yu, Y. J., J. B. Zhang, W. W. Chen, W. H. Zhong, T. B. Zhu and Z. C. Cai (2014). Effect of land use on the denitrification, abundance of denitrifiers, and total nitrogen gas production in the subtropical region of China. *Biology and Fertility of Soils* **50**(1): 105-113.

Zhang, A. F., L. Q. Cui, G. X. Pan, L. Q. Li, Q. Hussain, X. H. Zhang, J. W. Zheng and D. Crowley (2010). Effect of biochar amendment on yield and methane and nitrous oxide emissions from a rice paddy from Tai Lake plain, China. *Agriculture Ecosystems & Environment* **139**(4): 469-475.

Zheng, H., Z. Y. Wang, X. Deng, S. Herbert and B. S. Xing (2013). Impacts of adding biochar on nitrogen retention and bioavailability in agricultural soil. *Geoderma* **206**: 32-39.

Zumft, W. G. (1997). Cell biology and Molecular Basis of Denitrification. *Microbiology and Molecular Biology Reviews* **61**(4): 533-616.